P TENT COOPERATION TRE Y

From the INTERNATIONAL BUREAU

To:

Commissioner
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United States Patent and Trademark Office, PCT
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Arlington, VA 22202
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in its capacity as elected Office

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

Date of mailing (day/month/year) 30 May 2001 (30.05.01)	
International application No. PCT/GB00/03149	**Applicant's or agent's file reference** 44.68101/001
International filing date (day/month/year) 15 August 2000 (15.08.00)	**Priority date** (day/month/year) 19 August 1999 (19.08.99)
Applicant NILSEN, Jorunn et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

16 March 2001 (16.03.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

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Form PCT/IB/331 (July 1992)

GB0003149

PATENT COOPERATION TREATY

14

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 44.68101/001	**FOR FURTHER ACTION** See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/GB00/03149	International filing date *(day/month/year)* 15/08/2000	Priority date *(day/month/year)* 19/08/1999
International Patent Classification (IPC) or national classification and IPC B29C49/00		
Applicant BOREALIS TECHNOLOGY OY		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand 16/03/2001	Date of completion of this report 16.10.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Kujat, C Telephone No. +49 89 2399 2360

I. Basis f the r port

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-11	as originally filed			

Claims, No.:

1-7	as received on	01/10/2001	with letter of	26/09/2001

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:
   - ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-7
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-7
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-7
	No:	Claims	

2. Citations and explanations
   see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following document:
D1: WO 95 11264 A (MOBIL OIL CORP) 27 April 1995 (1995-04-27)

1.1 The subject-matter of independent process **claim 1** differs from the disclosure of document **D1** in that the HDPE contains an ethylene homopolymer and an ethylene copolymer.

1.2 The problem underlying the present invention may therefore be considered as providing a different bimodal HDPE for the preparation of at least 2L volume containers.

1.3 The characterizing portion of claim 1 hints at the use of a "tandem process" for the preparation of the bimodal HDPE. Although document D1 also briefly mentions that process in the introductory part of the description, D1 actually teaches away from a tandem process since document D1 focuses on a "single reactor" process. Hence, the bimodal HDPE of document D1 cannot contain both a homopolymer and a copolymer component, as specified in claim 1. Therefore, the subject-matter of **independent claim 1 is novel and involves an inventive step.**

1.4 **Claims 2 to 4 and 7** are dependent on claim 1 and as such also meet the requirements of the PCT with respect to novelty and inventive step.

1.5 Independent product **claim 5** is directed to the container made by the method of claim 1. This container itself is novel and inventive, since it is made of bimodal HDPE containing an ethylene homopolymer and an ethylene copolymer.

1.4 **Claim 6** is dependent on claim 5 and as such also meets the requirements of the PCT with respect to novelty and inventive step.

<u>Re Item VII</u>

Certain defects in the international application

2.1 Independent product **claim 5** is not in the **two-part form** in accordance with Rule 6.3(b) PCT, which in the present case would be appropriate, with those features known in combination from the prior art (document **D1**) being placed in the preamble (Rule 6.3(b)(i) PCT) and with the remaining features being included in the characterising part (Rule 6.3(b)(ii) PCT).

2.2 Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the document **D1** is not mentioned in the description, nor is this document identified therein.

PATENT COOPERATION TREATY

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
44.68101/001		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 00/03149	15/08/2000	19/08/1999
Applicant		
BOREALIS TECHNOLOGY OY		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 03 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.
   [ ] filed together with the international application in computer readable form.
   [ ] furnished subsequently to this Authority in written form.
   [ ] furnished subsequently to this Authority in computer readble form.
   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.
   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.
   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. ______

   [ ] as suggested by the applicant.
   [ ] because the applicant failed to suggest a figure.
   [ ] because this figure better characterizes the invention.
   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/03149

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 B29C49/00 //B29K23:00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 B29C C08F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 11264 A (MOBIL OIL CORP) 27 April 1995 (1995-04-27) page 17, line 18; claims 1,4,7,26; figure 1 ---	1-7
A	EP 0 757 076 A (UNION CARBIDE CHEM PLASTIC) 5 February 1997 (1997-02-05) example 4; table 2 ---	1,5,7
A	WO 99 40126 A (BOREALIS AS ;HOKKANEN HARRI (FI); ALMQUIST VIDAR (NO); FOLLESTAD A) 12 August 1999 (1999-08-12) page 4, line 1 - line 12; figure 1; tables 3-6 ---	1,5,7
A	WO 99 03899 A (MOBIL OIL CORP) 28 January 1999 (1999-01-28) page 1, paragraph 3 ---	1
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 October 2000	07/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van Nieuwenhuize, O

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 47682 A (SAHILA AIMO ;AEAERILAE JARI (FI); BOREALIS POLYMERS OY (FI); HAGST) 18 December 1997 (1997-12-18) claim 33 ---	1
A	WO 97 42237 A (DSM NV ;TOL MAURITS FREDERIK HENDRIK V (NL); BEEK JOHANNES ANTONIU) 13 November 1997 (1997-11-13) claim 21 ---	1
A	EP 0 339 571 A (SHOWA DENKO KK) 2 November 1989 (1989-11-02) page 2, line 23 - line 27 -----	1

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 00/03149

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 9511264	A	27-04-1995	AU	692192	B	04-06-1998
			AU	7983794	A	08-05-1995
			CA	2171019	A	27-04-1995
			EP	0724604	A	07-08-1996
			JP	9506912	T	08-07-1997
			US	5539076	A	23-07-1996
EP 0757076	A	05-02-1997	AU	6058196	A	30-01-1997
			BR	9603132	A	05-05-1998
			CA	2181607	A	22-01-1997
			CN	1152494	A	25-06-1997
			JP	9117949	A	06-05-1997
WO 9940126	A	12-08-1999	AU	2443099	A	23-08-1999
			AU	2443199	A	23-08-1999
			EP	1045866	A	25-10-2000
			WO	9940131	A	12-08-1999
WO 9903899	A	28-01-1999	US	6051525	A	18-04-2000
			AU	8280298	A	10-02-1999
			EP	0996648	A	03-05-2000
WO 9747682	A	18-12-1997	FI	962366	A	08-12-1997
			AU	3034997	A	07-01-1998
			AU	6308096	A	10-02-1997
			BR	9609604	A	25-05-1999
			CA	2226549	A	30-01-1997
			CN	1195363	A	07-10-1998
			EP	0837915	A	29-04-1998
			EP	0902809	A	24-03-1999
			WO	9703139	A	30-01-1997
			PL	325016	A	06-07-1998
WO 9742237	A	13-11-1997	AU	2411797	A	26-11-1997
EP 0339571	A	02-11-1989	JP	1272605	A	31-10-1989
			JP	2640491	B	13-08-1997
			JP	1292009	A	24-11-1989
			JP	2678914	B	19-11-1997
			DE	68913226	D	31-03-1994
			DE	68913226	T	29-09-1994
			NO	891710	A,B,	27-10-1989

CORRECTED VERSION*

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [6] **:**
C07K 14/705, C12N 5/10, 15/09, 15/11, 15/12, 15/62, G01N 33/566
A1

(11) International Publication Number: **WO 99/40114**

(43) International Publication Date: 12 August 1999 (12.08.99)

(21) International Application Number: PCT/US99/02361

(22) International Filing Date: 3 February 1999 (03.02.99)

(30) Priority Data:
60/073,767 5 February 1998 (05.02.98) US

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(74) Common Representative: MERCK & CO., INC.; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).

(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: NOVEL GABA$_B$ RECEPTOR DNA SEQUENCES

(57) Abstract

DNA encoding a novel human GABA$_B$ receptor subunit, HG20, as well as the protein encoded by the DNA, is provided. Also provided is DNA encoding a novel murine GABA$_B$ receptor subunit, GABA$_B$R1a, as well as the protein encoded by the DNA. Heterodimers of HG20 protein and GABA$_B$R1a protein that form a functional GABA$_B$ receptor are disclosed. Methods of identifying agonists and antagonists of the GABA$_B$ receptor are also provided.

FOR THE PURPOSES OF INFORMATION ONLY

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TITLE OF THE INVENTION
NOVEL $GABA_B$ RECEPTOR DNA SEQUENCES

CROSS-REFERENCE TO RELATED APPLICATIONS

Not applicable.

STATEMENT REGARDING FEDERALLY-SPONSORED R&D

Not applicable.

REFERENCE TO MICROFICHE APPENDIX

Not applicable.

FIELD OF THE INVENTION

The present invention is directed to a novel human DNA sequence encoding HG20, a subunit of the $GABA_B$ receptor, the protein encoded by the DNA, and uses thereof. The present invention also is directed to the murine $GABA_BR1a$ subunit of the $GABA_B$ receptor as well as to methods of combining an HG20 subunit with a $GABA_BR1a$ subunit to form a $GABA_B$ receptor having functional activity.

BACKGROUND OF THE INVENTION

Amino acids such as glutamic acid, γ-amino-butyric acid (GABA), and glycine are neurotransmitters that bind to specific receptors in the vertebrate nervous system and mediate synaptic transmission. Of these amino acids, GABA is the most widely distributed amino acid inhibitory neurotransmitter in the vertebrate central nervous system. The biological activities of GABA are mediated by three types of GABA receptors: ionotropic $GABA_A$ receptors, metabotropic $GABA_B$ receptors, and ionotropic $GABA_C$ receptors. Each type of receptor has its own characteristic molecular structure, pattern of gene expression, agonist and antagonist mediated pharmacological effects, and spectrum of physiological activities.

$GABA_A$ receptors mediate fast synaptic inhibition. They are heterooligomeric proteins (most likely pentamers) containing α, β, γ, and

perhaps δ, subunits that function as ligand-gated Cl^- channels and have binding sites for benzodiazepines, barbiturates, and neuroactive steroids. Bicuculline is a widely used antagonist of $GABA_A$ receptors. Bicuculline is selective for $GABA_A$ receptors in that it has no effect on $GABA_B$ or $GABA_C$ receptors. The expression of $GABA_A$ receptors has been observed in a variety of brain structures (see. *e.g.*, McKernan & Whiting, 1996, Trends Neurosci. 16:139-143; Sequier et al., 1988, Proc. Natl. Acad. Sci. USA 85:7815-7819).

$GABA_C$ receptors are ligand-gated Cl^- channels found in the vertebrate retina. They can be distinguished from $GABA_A$ and $GABA_B$ receptors in that they are insensitive to the $GABA_A$ receptor antagonist bicuculline and the $GABA_B$ receptor agonist (-)baclofen but are selectively activated by *cis*-4-aminocrotonic acid. $GABA_C$ receptors are composed of homooligomers of a category of GABA receptor subunits known as "ρ" subunits, the best-studied of which are ρ1 and ρ2. ρ1 and ρ2 share 74% amino acid sequence identity but are only about 30-38% identical in amino acid sequence when compared to $GABA_A$ receptor subunits. For a review of $GABA_C$ receptors, see Bormann & Feigenspan, 1995, Trends Neurosci. 18:515-518.

$GABA_B$ receptors play a role in the mediation of late inhibitory postsynaptic potentials (IPSPs). $GABA_B$ receptors belong to the superfamily of seven transmembrane-spanning G-protein coupled receptors that are coupled through G-proteins to neuronal K^+ or Ca^{++} channels. $GABA_B$ receptors are coupled through G-proteins to neuronal K^+ or Ca^{++} channels, and receptor activation increases K^+ or decreases Ca^{++} conductance and also inhibits or potentiates stimulated adenylyl cyclase activity. The expression of $GABA_B$ receptors is widely distributed in the mammalian brain (*e.g.*, frontal cortex, cerebellar molecular layer, interpeduncular nucleus) and has been observed in many peripheral organs as well.

A large number of pharmacological activities have been attributed to $GABA_B$ receptor activation, *e.g.*, analgesia; hypothermia; catatonia; hypotension; reduction of memory consolidation and retention; and stimulation of insulin, growth hormone, and glucagon release (see

Bowery, 1989, Trends Pharmacol. Sci. 10:401-407, for a review.) It is well accepted that $GABA_B$ receptor agonists and antagonists are pharmacologically useful. For example, the $GABA_B$ receptor agonist (-)baclofen, a structural analog of GABA, is a clinically effective muscle relaxant (Bowery & Pratt, 1992, Arzneim.-Forsch./Drug Res. 42:215-223). (-)baclofen, as part of a racemic mixture with (+)baclofen, has been sold in the United States as a muscle relaxant under the name LIORESAL® since 1972.

$GABA_B$ receptors represent a large family of related proteins, new family members of which are still being discovered. For example, Kaupmann et al., 1997, Nature 386:239-246 (Kaupmann) reported the cloning and expression of two members of the rat $GABA_B$ receptor family, $GABA_B$R1a and $GABA_B$R1b. A variety of experiments using known agonists and antagonists of $GABA_B$ receptors seemed to indicate that $GABA_B$R1a and $GABA_B$R1b represented rat $GABA_B$ receptors. This conclusion was based primarily on the ability of $GABA_B$R1a and $GABA_B$R1b to bind agonists and antagonist of $GABA_B$ receptors with the expected rank order, based upon studies of rat cerebral cortex $GABA_B$ receptors. However, there were data that did not fit the theory that Kaupmann had cloned the pharmacologically and functionally active $GABA_B$ receptor. For example, Kaupmann noted that agonists had significantly lower binding affinity to recombinant $GABA_B$R1a and $GABA_B$R1b as opposed to native $GABA_B$ receptors. Also, Couve et al., 1998, J. Biol. Chem. 273:26361-26367 showed that recombinantly expressed $GABA_B$R1a and $GABA_B$R1b failed to target correctly to the plasma membrane and failed to give rise to functional $GABA_B$ receptors when expressed in a variety of cell types.

Examination of the amino acid and gene sequence of $GABA_B$R1a led Kaupmann to propose a structure for $GABA_B$R1a similar to that of the metabotropic glutamate receptor gene family. The metabotropic glutamate receptor family comprises eight glutamate binding receptors and five calcium sensing receptors which exhibit a signal peptide sequence followed by a large N-terminal domain believed to represent the ligand binding pocket that precedes seven transmembrane

spanning domains. The hallmark seven transmembrane spanning domains are typical of G-protein coupled receptors (GPCRs), although metabotropic glutamate receptors and $GABA_B$R1a are considerably larger than most GPCRs and contain a signal peptide sequence. No significant amino acid sequence similarities were found between $GABA_B$R1a and $GABA_A$ receptors, $GABA_C$ receptors, or other typical GPCRs.

Despite work such as that of Kaupmann, pharmacological and physiological evidence indicates that a large number of amino acid binding $GABA_B$ receptors remain to be cloned and expressed in recombinant systems where agonists and antagonists can be efficiently identified. In particular, it would be extremely valuable to be able to recombinantly express $GABA_B$ receptors in such a manner that not only pharmacologically relevant ligand binding properties would be exhibited by the recombinant receptors, but also such that the recombinant receptors would show proper functional activity.

SUMMARY OF THE INVENTION

The present invention is directed to a novel human DNA that encodes a $GABA_B$ receptor subunit, HG20. The DNA encoding HG20 is substantially free from other nucleic acids and has the nucleotide sequence shown in SEQ.ID.NO.:1. Also provided is an HG20 protein encoded by the novel DNA sequence. The HG20 protein is substantially free from other proteins and has the amino acid sequence shown in SEQ.ID.NO.:2. Methods of expressing HG20 in recombinant systems and of identifying agonists and antagonists of HG20 are provided.

The present invention is also directed to a novel murine DNA that encodes a $GABA_B$ receptor subunit, $GABA_B$R1a. The DNA encoding $GABA_B$R1a is substantially free from other nucleic acids and has the nucleotide sequence shown in SEQ.ID.NO.:19. Also provided is a $GABA_B$R1a protein encoded by the novel DNA sequence. The $GABA_B$R1a protein is substantially free from other proteins and has the amino acid sequence shown in SEQ.ID.NO.:20. Methods of expressing $GABA_B$R1a in recombinant systems and of identifying agonists and antagonists of HG20 are provided.

Also provided by the present invention are methods of co-expressing HG20 and $GABA_BR1a$ in the same cells. Such co-expression results in the production of a $GABA_B$ receptor that exhibits expected functional properties of $GABA_B$ receptors as well as expected ligand binding properties. Recombinant cells co-expressing HG20 and $GABA_BR1a$ are provided as well as methods of utilizing such recombinant cells to identify agonists and antagonists of $GABA_B$ receptors.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-B shows the complete cDNA sequence of HG20 (SEQ.ID.NO.:1).

Figure 2 shows the complete amino acid sequence of HG20 (SEQ.ID.NO.:2).

Figure 3A-B shows predicted signal peptide cleavage sites of HG20. All sequences shown are portions of SEQ.ID.NO.:2.

Figure 4 shows *in situ* analysis of the expression of HG20 RNA in squirrel monkey brain.

Figure 5A shows *in vitro* coupled transcription/translation of a chimeric FLAG epitope-HG20 (amino acids 52-941) protein.

Figure 5B shows the expression in COS-7 cells and melanophores of a chimeric FLAG epitope-HG20 (amino acids 52-941) protein.

Figure 6 shows a comparison of the amino acid sequences of a portion of the N-terminus of HG20 protein and the ligand binding domain of the *Pseudomonas aeruginosa* amino acid binding protein LIVAT-BP (Swiss Protein database accession number P21175). The upper sequence shown is from HG20 and corresponds to amino acids 63-259 of SEQ.ID.NO.:2. The lower sequence shown is from *Pseudomonas aeruginosa* LIVAT-BP and is SEQ.ID.NO.:16.

Figure 7 shows expression in mammalian cells of a chimeric HG20 protein.

Figure 8 shows a comparison of the amino acid sequences of HG20 and $GABA_BR1b$. The HG20 sequence is SEQ.ID.NO.:2. The $GABA_BR1b$ sequence is SEQ.ID.NO.:17.

Figure 9 shows the expression of recombinant $GABA_BR1a$ and HG20 in COS-7 cells. Lanes 1 and 2 show $[^{125}I]$CGP71872 photolabeling of recombinant murine $GABA_BR1a$ monomer and dimer in the presence (+) and absence (-) of 1 μM unlabeled CGP71872. Lanes 3 and 4 show that $GABA_BR1a$ antibodies 1713.1-1713.2 confirmed (+) expression of recombinantly expressed murine $GABA_BR1a$ (referred to as mgb1a here) and absence (-) in pcDNA3.1 mock transfected cells. Lanes 5 and 6 show $[^{125}I]$CGP71872 photolabeling of human FLAG-HG20 in the presence (+) and absence (-) of 1 μM unlabeled CGP71872. Lanes 7 and 8 show that an anti-FLAG antibody confirmed (+) the expression of FLAG-HG20 (referred to as FLAG-gb2 here) and its absence (-) in pcDNA3.1 mock transfected cells. Experimental details were as in Examples 7-9 and 20 except that COS-7 rather than COS-1 cells were used.

Figure 10 shows co-localization of mRNA for HG20 and $GABA_BR1a$ by *in situ* hybridization histochemistry in rat parietal cortex. Adjacent coronal sections of rat brain showing parietal cortex hybridized with radiolabelled $GABA_BR1a$ (A) and HG20 (B) probes. Rat $GABA_BR1a$ and HG20 probes were labelled using ^{35}S-UTP (A, B, and D), and autoradiograms were developed after 4 weeks. For co-localization studies, the rat $GABA_BR1a$ probe was digoxigenin labelled and developed using anti-digoxigenin HRP, the TSA amplification method and biotinyl tyramide followed by streptavidin-conjugated CY3 (C). (D) shows autoradiography of the same field as in (C), denoting hybridization to HG20 mRNA. (E) is an overlay of images (C) and (D). Arrows denote some of the double-labelled cells. Scale bar = 0.5 mm in (A) and (B); scale bar = 50 um in (C-E).

Figure 11 shows functional complementation following co-expression of $GABA_BR1a$ and HG20 in *Xenopus* melanophores. GABA mediated a dose-dependent aggregation response in melanophores co-expressing murine $GABA_BR1a$ and FLAG-HG20 (■) that could be blocked with 100 nM (▼) and 1 μM CGP71872 (▲). The response of GABA on mock-transfected cells is shown (●) as well as a control cannabinoid receptor subtype 2 response to HU210 ligand (inset). This experiment is representative of n=4.

Figure 12 shows GABA$_B$ receptor modulation of forskolin-stimulated cAMP synthesis in HEK293 cells. HEK293 cells stably expressing HG20 (hgb2-42) or GABA$_B$R1a (rgb1a-50) were transiently transfected with GABA$_B$R1a and HG20 expression plasmids to examine the effect of receptor co-expression on modulation of cAMP synthesis. All transfected cells were tested with 300 μM baclofen or GABA (with 100 μM AOAA and 100 μM nipecotic acid) in the absence of forskolin and 30 μM baclofen or GABA in the presence of 10 μM forskolin. Wild-type HEK293 cells were tested with 250 μM baclofen or 250 μM GABA in the presence of 10 μM forskolin. Data are presented as the percent of total cAMP synthesized in the presence of forskolin only. The data presented are from single representative experiments that have been replicated twice. Fsk, forskolin; B, baclofen; G, GABA with AOAA and nipecotic acid. The two right-most set of bar graphs (labeled "B + Fsk" and "G + Fsk") show that in cells expressing both GABA$_B$R1a and HG20 (rgb1a-50/hgb2 cells (❑) and hgb2-42/rgb1a cells (■)), baclofen and GABA were able to mediate significant reductions in cAMP levels.

Figure 13 shows that co-expression of GABA$_B$R1a and HG20 permits inwardly rectifying potassium channel (GIRK or Kir) activation in Xenopus oocytes. (A) Representative current families of Kir 3.1/3.2. Currents were evoked by 500 msec voltage commands from a holding potential of -10 mV, delivered in 20 mV increments from -140 to 60 mV. (B) In a protocol designed to measure the effects of various receptors on Kir currents, oocytes were held at -80 mV (a potential where significant inward current is measured). Expression of GABA$_B$R1a or HG20 alone (with or without Giα1) resulted in no modulation of current after GABA treatment. Co-expression of GABA$_B$R1a and FLAG-HG20 receptors followed by treatment with 100 μM GABA resulted in stimulation of Kir 3.1/3.2. Shown are representative traces from at least three independent experiments under each condition.

Figure 14 shows immunoblotting of murine GABA$_B$R1a and FLAG-HG20 transiently expressed in COS-7 cells. Digitonin-solubilized and anti-FLAG antibody immunoprecipitated membrane proteins were immunoblotted following SDS-PAGE with GABA$_B$R1a antibodies 1713.1-

1713.2. The conditions are as follows: mock pcDNA3.1 vector transfected cells (lane 1), FLAG-HG20 expressing cells (lane 2), murine GABA$_B$R1a expressing cells (lane 3), and cells coexpressing murine GABA$_B$R1a and FLAG-HG20 (lane 4). The immunoreactive band corresponding to the GABA$_B$R1a /HG20 heterodimer as well as a band corresponding to the predicted GABA$_B$R1a monomer are denoted by arrows.

Figure 15 shows the complete cDNA sequence of murine GABA$_B$R1a (SEQ.ID.NO.:19). The sequence shown has been deposited in GenBank (accession number AF114168).

Figure 16 shows the complete amino acid sequence of murine GABA$_B$R1a (SEQ.ID.NO.:20). The sequence shown has been deposited in GenBank (accession number AF114168).

Figure 17A-B shows the results of experiments with N- and C-terminal fragments of murine GABA$_B$R1a. Figure 17A shows the results of coupled *in vitro* transcription/translation reactions; lane 1 = blank; lane 2 = full-length GABA$_B$R1a; lane 3 = N-terminal fragment of GABA$_B$R1a; lane 4 = C-terminal fragment of GABA$_B$R1a. Figure 17B shows the results of [^{125}I]CGP71872 photoaffinity labeling; lane 1 = N-terminal fragment of GABA$_B$R1a; lane 2 = N-terminal fragment of GABA$_B$R1a in the presence of GABA; lane 3 = C-terminal fragment of GABA$_B$R1a; lane 4 = C-terminal fragment of GABA$_B$R1a in the presence of GABA.

Figure 18A-B shows the amino acid sequence (Figure 18A) (SEQ.ID.NO.:21) and nucleotide sequence (Figure 18B) (SEQ.ID.NO.:22) (GenBank accession number AJ012185) of a human GABA$_B$R1a.

Figure 19A-B shows the nucleotide sequence (SEQ.ID.NO.:23) (GenBank accession number Y11044) of a human GABA$_B$R1a.

Figure 20 shows a framework map of chromosome 9. The locations of the HG20 gene (referred to as "GPR 51"), markers, and the HSN-1 locus are indicated.

Figure 21 shows a hydropathy plot for murine GABA$_B$R1a.

Figure 22 shows a family tree of genes related to HG20. Abbreviations are as follows: hGB1a = human GABA$_B$R1a; mGB1a =

mouse GABA$_B$R1a; rGB1a = rat GABA$_B$R1a; hGB1b = human GABA$_B$R1b; rGB1b = rat GABA$_B$R1b; ceGB1b = a *C. elegans* gene related to mammalian GABA$_B$R1a and GABA$_B$R1b; hGB2 = human HG20; ceGB2 = a *C. elegans* gene related to human HG20; MGRDROME = a metabotropic glutamate receptor from *Drosophila melanogaster*; MGR2 HUMAN = human metabotropic glutamate receptor 2; MGR3 HUMAN = human metabotropic glutamate receptor 3; MGR6 HUMAN = human metabotropic glutamate receptor 6; MGR4 HUMAN = human metabotropic glutamate receptor 4; MGR7 HUMAN = human metabotropic glutamate receptor 7; MGR8 HUMAN = human metabotropic glutamate receptor 8; MGR1 HUMAN = human metabotropic glutamate receptor 1; MGR5 HUMAN = human metabotropic glutamate receptor 5.

Figure 23 shows the coiled-coil domains in the C-termini of human GABA$_B$R1a and HG20. The upper sequence is from human GABA$_B$R1a and is positions 886-949 of SEQ.ID.NO.:21. The lower sequence is from HG20 and is positions 756-829 of SEQ.ID.NO.:2.

Figure 24 shows a comparison of the amino acid sequences of human GABA$_B$R1a (referred to as "Human GABA-B1aR," SEQ.ID.NO.:21); the proteins encoded by two genes from C. elegans (*C. elegans* GABA-B1 = SEQ.ID.NO.:42 and *C. elegans* GABA-B2 = SEQ.ID.NO.:43); and HG20) (referred to as "Human GABA-B2," (SEQ.ID.NO.:2). The *C. elegans* genes have been predicted from *C.elegans* DNA sequence alone. ZK180 accession number: U58748 is predicted to be GABA-B2 and Y41G9. Contig99 and Y76F7.Contig73 were obtained from the Sanger *C. elegans* genomic sequence database and are predicted to be GABA-B1.

Figure 25A-D shows co-immunoprecipitation of the murine GABA$_B$R1a and FLAG-HG20 receptor subunits and immunoblotting using reciprocal receptor subunit antibodies. Murine GABA$_B$R1a and FLAG-HG20 receptors were expressed individually or co-expressed in COS-7 cells. Figure 25A shows the results of immunoblotting using an anti-murine GABA$_B$R1a antibody. Immunoblot of the solubilized membranes using murine GABA$_B$R1a antibodies 1713.1-1713.2 shows

selective expression of murine $GABA_BR1a$ in murine $GABA_BR1a$ alone expressing cells (lane 3) and murine $GABA_BR1a$ /FLAG-HG20 co-expressing cells (lane 4), but not in mock transfected and FLAG-HG20 alone expressing cells (lanes 1 and 2). Staining of $GABA_BR1a$ subunits in co-expressing cells is more intense compared to cells expressing the $GABA_BR1a$ subunit alone, suggesting that HG20 subunits facilitate $GABA_BR1a$ expression. Figure 25B shows the results of immunoblotting using an anti-FLAG-HG20 antibody. Immunoblotting of the solubilized membranes using the anti-FLAG-HG20 antibody shows selective expression of FLAG-HG20 subunits in FLAG-HG20 alone expressing cells (lane 6) and murine $GABA_BR1a$ /FLAG-HG20 co-expressing cells (lane 8), but not in mock transfected and murine $GABA_BR1a$ alone expressing cells (lanes 5 and 7). Staining of HG20 subunits in co-expressing cells is more intense compared to cells expressing the HG20 subunit alone, suggesting that $GABA_BR1a$ subunits facilitate HG20 expression. Figure 25C shows the results of immunoprecipitation with an anti-FLAG-HG20 antibody followed by immunoblotting with an anti-murine $GABA_BR1a$ antibody. $GABA_BR1a$ /HG20 heterodimers are observed only in murine $GABA_BR1a$ /FLAG-HG20 co-expressing cells due to the fact that the $GABA_BR1a$ subunit was co-immunoprecipitated with the FLAG-HG20 subunit using the FLAG antibody and detected with $GABA_BR1a$ antibodies (lane 12). $GABA_BR1a$ subunits are not detected in mock-transfected cells and cells expressing $GABA_BR1a$ alone or FLAG-HG20 (lanes 9-11). Figure 25D shows the results of immunoprecipitation with an anti-murine $GABA_BR1a$ antibody followed by immunoblotting with an anti-FLAG-HG20 antibody. $GABA_BR1a$ /HG20 heterodimers are observed only in murine $GABA_BR1a$ /FLAG-HG20 co-expressing cells due to the fact that the FLAG-HG20 subunit was co-immunoprecipitated using the $GABA_BR1a$ antibodies and detected with FLAG antibody (lane 16). No FLAG-HG20 subunits are detected in mock-transfected cells or cells expressing murine $GABA_BR1a$ alone or FLAG-HG20 (lanes 13-15). The immunoblots shown are from 1-3 independent experiments.

Figure 26A-B shows some of the motifs in the N-termini of $GABA_B$ receptor subunits and related genes. Figure 26A shows an

alignment of murine $GABA_BR1a$ (mGABAb1a; a portion of SEQ.ID.NO.:20), human $GABA_BR1a$ (hGABAb1a; a portion of SEQ.ID.NO.:21), HG20 (hGABAb2; a portion of SEQ.ID.NO.:2), metabotropic glutamate receptor 1 (mGluR1; SEQ.ID.NO.:44), and two *E. coli* proteins (LivK (SEQ.ID.NO.:45) and LivBP (SEQ.ID.NO.:46)). Figure 26B is a schematic drawing showing the location of the various motifs in murine $GABA_BR1a$ that are expected to be involved in heterodimer formation of $GABA_BR1a$ with HG20.

Figure 27 shows an expanded view of the coiled-coil region of homology between HG20 (hGABAb2; shown is a portion of SEQ.ID.NO.:2) and murine $GABA_BR1a$ (mGABAb1a; a portion of SEQ.ID.NO.:20). Also shown is the corresponding region of human $GABA_BR1a$ (hGABAb1a; a portion of SEQ.ID.NO.:21).

DETAILED DESCRIPTION OF THE INVENTION

For the purposes of this invention:

"Substantially free from other proteins" means at least 90%, preferably 95%, more preferably 99%, and even more preferably 99.9%, free of other proteins. Thus, for example, an HG20 protein preparation that is substantially free from other proteins will contain, as a percent of its total protein, no more than 10%, preferably no more than 5%, more preferably no more than 1%, and even more preferably no more than 0.1%, of non-HG20 proteins. Whether a given HG20 protein preparation is substantially free from other proteins can be determined by such conventional techniques of assessing protein purity as, *e.g.*, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) combined with appropriate detection methods, *e.g.*, silver staining or immunoblotting.

"Substantially free from other nucleic acids" means at least 90%, preferably 95%, more preferably 99%, and even more preferably 99.9%, free of other nucleic acids. Thus, for example, an HG20 DNA preparation that is substantially free from other nucleic acids will contain, as a percent of its total nucleic acid, no more than 10%, preferably no more than 5%, more preferably no more than 1%, and even more

preferably no more than 0.1%, of non-HG20 nucleic acids. Whether a given HG20 DNA preparation is substantially free from other nucleic acids can be determined by such conventional techniques of assessing nucleic acid purity as, *e.g.*, agarose gel electrophoresis combined with appropriate staining methods, *e.g.*. ethidium bromide staining, or by sequencing.

An HG20 polypeptide has "substantially the same biological activity" as native HG20 (*i.e.*, SEQ.ID.NO.:2) if that polypeptide has a K_d for a ligand that is no more than 5-fold greater than the K_d of native HG20 for the same ligand. An HG20 polypeptide also has "substantially the same biological activity" as HG20 if that polypeptide can form heterodimers with either a $GABA_BR1a$ or $GABA_BR1b$ polypeptide, thus forming a functional $GABA_B$ receptor.

"Functional $GABA_B$ receptor" refers to a heterodimer of HG20 and either $GABA_BR1a$ or $GABA_BR1b$ where the heterodimer displays a functional response when exposed to GABA agonists. Examples of functional responses are: pigment aggregation in *Xenopus* melanophores, modulation of cAMP levels, coupling to inwardly rectifying potassium channels, mediation of late inhibitory postsynaptic potentials in neurons, increase in potassium conductance, and decrease in calcium conductance. One skilled in the art would be familiar with a variety of methods of measuring the functional responses of G-protein coupled receptors such as the $GABA_B$ receptor (see, *e.g.*, Lerner, 1994, Trends Neurosci. 17:142-146 [changes in pigment distribution in melanophore cells]; Yokomizo et al., 1997, Nature 387:620-624 [changes in cAMP or calcium concentration; chemotaxis]; Howard et al., 1996, Science 273:974-977 [changes in membrane currents in *Xenopus* oocytes]; McKee et al., 1997, Mol. Endocrinol. 11:415-423 [changes in calcium concentration measured using the aequorin assay]; Offermanns & Simon, 1995, J. Biol. Chem. 270:15175, 15180 [changes in inositol phosphate levels]). Depending upon the cells in which heterodimers of HG20 and either $GABA_BR1a$ or $GABA_BR1b$ are expressed, and thus the G-proteins with which the heterodimers are coupled, certain of such methods may be appropriate for measuring the functional responses of such heterodimers.

It is well with the competence of one skilled in the art to select the appropriate method of measuring functional responses for a given experimental system.

A $GABA_BR1a$ or $GABA_BR1b$ polypeptide has "substantially the same biological activity" as a native $GABA_BR1a$ or $GABA_BR1b$ polypeptide if that polypeptide has a K_d for an amino acid, amino acid analogue, $GABA_B$ receptor agonist, or $GABA_B$ receptor antagonist such as CGP71872, GABA, saclofen, (-)baclofen, or (L)-glutamic acid that is no more than 5-fold greater than the K_d of a native $GABA_BR1a$ or $GABA_BR1b$ polypeptide for the same amino acid, amino acid analogue, $GABA_B$ receptor agonist, or $GABA_B$ receptor antagonist. A $GABA_BR1a$ or $GABA_BR1b$ polypeptide also has "substantially the same biological activity" as a native $GABA_BR1a$ or $GABA_BR1b$ polypeptide if that polypeptide can form heterodimers with an HG20 polypeptide, thus forming a functional $GABA_B$ receptor. Native $GABA_BR1a$ or $GABA_BR1b$ polypeptides include the murine $GABA_BR1a$ sequence shown as SEQ.ID.NO.:20; the rat $GABA_BR1a$ or $GABA_BR1b$ polypeptides disclosed in Kaupmann et al., 1997, Nature 386:239-246; the human $GABA_BR1a$ sequence disclosed in GenBank accession number AJ012185 (SEQ.ID.NO.:21); and the protein encoded by the DNA sequence disclosed in GenBank accession number Y11044 (SEQ.ID.NO.:23).

A "conservative amino acid substitution" refers to the replacement of one amino acid residue by another, chemically similar, amino acid residue. Examples of such conservative substitutions are: substitution of one hydrophobic residue (isoleucine, leucine, valine, or methionine) for another; substitution of one polar residue for another polar residue of the same charge (*e.g.*, arginine for lysine; glutamic acid for aspartic acid).

The present invention relates to the identification and cloning of HG20, a novel G-protein coupled receptor-like protein that represents a subunit for the $GABA_B$ receptor. The present invention provides DNA encoding HG20 that is substantially free from other nucleic acids. The present invention also provides recombinant DNA molecules encoding HG20 as well as isolated DNA molecules encoding HG20.

Following the cloning of HG20 by the present inventors, a sequence highly similar to the sequence of HG20 was deposited in GenBank by Clark et al. (GenBank accession number AF056085), by White et al. (GenBank accession number AJ012188), and by Borowsky et al. (GenBank accession number AF074483). Two ESTs (GenBank accession number T07621, deposited June 30, 1993, and GenBank accession number Z43654, deposited September 21, 1995) each contain partial sequences of HG20 cDNA.

The present invention provides a DNA molecule substantially free from other nucleic acids comprising the nucleotide sequence shown in Figure 1 as SEQ.ID.NO.:1. Analysis of SEQ.ID.NO.:1 revealed that it contains a long open reading frame at positions 293-3,115. Thus, the present invention also provides a DNA molecule substantially free from other nucleic acids comprising the nucleotide sequence of positions 293-3,115 of SEQ.ID.NO.:1. The present invention also provides an isolated DNA molecule comprising the nucleotide sequence of positions 293-3,115 of SEQ.ID.NO.:1.

Sequence analysis of the open reading frame of the HG20 DNA revealed that it encodes a protein of 941 amino acids with a calculated molecular weight of 104 kd and a predicted signal peptide. The predicted amino acid sequence of HG20 is 36% identical to the metabotropic GABA receptor-like sequence $GABA_BR1a$ described in Kaupmann (see above) throughout the entire sequence, and thus HG20 most likely represents a novel metabotropic GABA receptor or receptor subunit. *In situ* hybridization showed that HG20 RNA is highly expressed in the cortex, thalamus, hippocampus, and cerebellum of the brain, showing overlapping distribution with $GABA_BR1a$ RNA as judged by *in situ* hybridization as well as with the expression of $GABA_B$ receptors as judged by pharmacological studies. HG20 RNA exhibits restricted distribution in the periphery, with low abundance of the 6.5 kb RNA in the heart, spleen, and pancreas and high levels in the adrenal gland. HG20 recombinantly expressed in COS-1 cells showed no specific binding for $[^3H]$(+)baclofen, and when expressed in *Xenopus* oocyte and *Xenopus* melanophore functional assays, showed no activity to GABA,

(-)baclofen, and glutamic acid.

The novel DNA sequences of the present invention encoding HG20, in whole or in part, can be linked with other DNA sequences, *i.e.*, DNA sequences to which HG20 is not naturally linked, to form "recombinant DNA molecules" containing HG20. Such other sequences can include DNA sequences that control transcription or translation such as, *e.g.*, translation initiation sequences, promoters for RNA polymerase II, transcription or translation termination sequences, enhancer sequences, sequences that control replication in microorganisms, or that confer antibiotic resistance. The novel DNA sequences of the present invention can be inserted into vectors such as plasmids, cosmids, viral vectors, or yeast artificial chromosomes.

The present invention also includes isolated forms of DNA encoding HG20. By "isolated DNA encoding HG20" is meant DNA encoding HG20 that has been isolated from a natural source or produced by recombinant means. Use of the term "isolated" indicates that DNA encoding HG20 is not present in its normal cellular environment. Thus, an isolated DNA encoding HG20 may be in a cell-free solution or placed in a different cellular environment from that in which it occurs naturally. The term isolated does not imply that isolated DNA encoding HG20 is the only DNA present. but instead means that isolated DNA encoding HG20 is at least 95% free of non-nucleic acid material (*e.g.*, proteins, lipids, carbohydrates) naturally associated with the DNA encoding HG20. Thus, DNA encoding HG20 that is expressed in bacteria or even in eukaryotic cells which do not naturally (*i.e.*, without human intervention) contain it through recombinant means is "isolated DNA encoding HG20."

Included in the present invention are DNA sequences that hybridize to SEQ.ID.NO.:1 under stringent conditions. By way of example, and not limitation, a procedure using conditions of high stringency is as follows: Prehybridization of filters containing DNA is carried out for 2 hr. to overnight at 65°C in buffer composed of 6X SSC, 5X Denhardt's solution, and 100 μg/ml denatured salmon sperm DNA. Filters are hybridized for 12 to 48 hrs at 65°C in prehybridization mixture containing 100 μg/ml denatured salmon sperm DNA and 5-20 X 10^6 cpm

of ^{32}P-labeled probe. Washing of filters is done at 37°C for 1 hr in a solution containing 2X SSC, 0.1% SDS. This is followed by a wash in 0.1X SSC, 0.1% SDS at 50°C for 45 min. before autoradiography.

Other procedures using conditions of high stringency would include either a hybridization carried out in 5XSSC, 5X Denhardt's solution, 50% formamide at 42°C for 12 to 48 hours or a washing step carried out in 0.2X SSPE, 0.2% SDS at 65°C for 30 to 60 minutes.

Reagents mentioned in the foregoing procedures for carrying out high stringency hybridization are well known in the art. Details of the composition of these reagents can be found in, *e.g.*, Sambrook, Fritsch, and Maniatis, 1989, Molecular Cloning: A Laboratory Manual, second edition, Cold Spring Harbor Laboratory Press. In addition to the foregoing, other conditions of high stringency which may be used are well known in the art.

Another aspect of the present invention includes host cells that have been engineered to contain and/or express DNA sequences encoding HG20. Such recombinant host cells can be cultured under suitable conditions to produce HG20. An expression vector containing DNA encoding HG20 can be used for expression of HG20 in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to, bacteria such as *E. coli*, fungal cells such as yeast, mammalian cells including, but not limited to, cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to *Drosophila* and silkworm derived cell lines. Cell lines derived from mammalian species which are suitable for recombinant expression of HG20 and which are commercially available, include but are not limited to, L cells L-M(TK$^-$) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), HEK293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), *Xenopus* melanophores, and *Xenopus* oocytes. In particular embodiments, the recombinant cells expressing HG20 protein co-express a $GABA_BR1a$ or $GABA_BR1b$ protein, thus forming a functional $GABA_B$

receptor comprising a heterodimer of HG20 and either GABA$_B$R1a or GABA$_B$R1b. In partiular embodiments, the recombinant cells have been transfected with expression vectors that direct the expression of HG20 and GABA$_B$R1a or GABA$_B$R1b.

Cells that are particularly suitable for expression of the HG20 protein are melanophore pigment cells from *Xenopus laevis*. Such melanophore pigment cells can be used for functional assays that employ recombinant expression of HG20 in a manner similar to the use of such melanophore pigment cells for the functional assay of other recombinant GPCRs (Graminski et al., 1993, J. Biol. Chem. 268:5957-5964; Lerner, 1994, Trends Neurosci. 17:142-146; Potenza & Lerner, 1992, Pigment Cell Res. 5:372-378; Potenza et al., 1992, Anal. Biochem. 206:315-322). Especially preferred are *Xenopus* melanophore pigment cells co-expressing HG20 and GABA$_B$R1a or GABA$_B$R1b, in which HG20 has formed a heterodimer with GABA$_B$R1a or GABA$_B$R1b, thus forming a functional GABA$_B$ receptor. The presence of functional GABA$_B$ receptors in such cells can be determined by the use of assays such as the pigment aggregation assay described herein. Other assays that reflect a decrease in cAMP levels mediated by exposure to GABA or other agonists of GABA$_B$ receptors would also be suitable.

Also preferred are stably or transiently transfected HEK293 cells co-expressing HG20 and GABA$_B$R1a or GABA$_B$R1b, in which HG20 has formed a heterodimer with GABA$_B$R1a or GABA$_B$R1b, thus forming a functional GABA$_B$ receptor. The presence of functional GABA$_B$ receptors in such cells can be determined by the use of assays such as those that measure cAMP levels as described herein.

Also preferred are *Xenopus* oocytes co-expressing HG20 and GABA$_B$R1a or GABA$_B$R1b, in which HG20 has formed a heterodimer with GABA$_B$R1a or GABA$_B$R1b, thus forming a functional GABA$_B$ receptor. The presence of functional GABA$_B$ receptors in such cells can be determined by the use of assays that measure coupling of functional GABA$_B$ receptors comprising heterodimers of HG20 and GABA$_B$R1a or GABA$_B$R1b to inwardly rectifying potassium channels (especially the Kir3 family).

In order to produce the above-described cells co-expressing HG20 and $GABA_BR1a$ or $GABA_BR1b$, expression vectors comprising DNA encoding HG20 and $GABA_BR1a$ or $GABA_BR1b$ can be transfected into the cells. HG20 and $GABA_BR1a$ or $GABA_BR1b$ can be transfected separately, each on its own expression vector, or, alternatively, a single expression vector encoding both HG20 and $GABA_BR1a$ or $GABA_BR1b$ can be used.

A variety of mammalian expression vectors can be used to express recombinant HG20, $GABA_BR1a$, or $GABA_BR1b$ in mammalian cells. Commercially available mammalian expression vectors which are suitable include, but are not limited to, pMC1neo (Stratagene), pSG5 (Stratagene), pcDNAI and pcDNAIamp, pcDNA3, pcDNA3.1, pCR3.1 (Invitrogen), EBO-pSV2-neo (ATCC 37593), pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), and the PT7TS oocyte expression vector (or similar expression vectors containing the globin 5' UTR and the globin 3' UTR). The choice of vector will depend upon cell type used, level of expression desired, and the like. Following expression in recombinant cells, HG20, $GABA_BR1a$, $GABA_BR1b$, or heterodimers of HG20 and either $GABA_BR1a$ or $GABA_BR1b$ can be purified to a level that is substantially free from other proteins by conventional techniques, *e.g.*, salt fractionation, ion exchange chromatography, size exclusion chromatography, hydroxylapatite adsorption chromatography, hydrophobic interaction chromatography, and preparative gel electrophoresis. Also, membrane preparations comprising HG20, $GABA_BR1a$, $GABA_BR1b$, or heterodimers of HG20 and either $GABA_BR1a$ or $GABA_BR1b$ can be prepared. Especially preferred are membrane preparations that comprise heterodimers of HG20 and either $GABA_BR1a$ or $GABA_BR1b$ in which the heterodimers represent functional $GABA_B$ receptors.

The present invention includes a method of producing HG20 protein comprising:

(a) transfecting a host cell with an expression vector comprising DNA that encodes an HG20 protein;

(b) growing the host cells under conditions such that HG20 protein is produced; and

(c) recovering HG20 protein from the host cells.

In particular embodiments, the method of recovering HG20 protein involves obtaining membrane preparations that contain HG20 protein from the host cells. In particular embodiments, such membrane preparations contain heterodimers of HG20 protein and $GABA_BR1a$ or $GABA_BR1b$ protein that form functional $GABA_B$ receptors.

The present invention includes a method of expressing a truncated HG20 protein comprising:

(a) transfecting a host cell with an expression vector comprising DNA that encodes an HG20 protein that has been truncated at the amino or carboxyl terminus;

(b) culturing the transfected cells of step (a) under conditions such that the truncated HG20 protein is expressed.

Truncated HG20 proteins are those HG20 proteins in which contiguous portions of the N terminus or C terminus have been removed. For example, positions 52-941 of SEQ.ID.NO.:2 represents a truncated HG20 protein. Truncated HG20 proteins may be fused in frame to non-HG20 amino acid sequences, as, *e.g.*, in the FLAG-HG20 construct described herein.

The present invention includes a method of producing functional $GABA_B$ receptors in cells comprising:

(a) transfecting cells with:

(1) an expression vector that directs the expression of HG20 in the cells; and

(2) an expression vector that directs the expression of $GABA_BR1a$ or $GABA_BR1b$ in the cells;

(b) culturing the cells under conditions such that heterodimers of HG20 and $GABA_BR1a$ or $GABA_BR1b$ are formed where the heterodimers constitue functional $GABA_B$ receptors.

In particular embodiments of the above methods, the cells are eukaryotic cells. In other embodiments, the cells are mammalian cells. In still other embodiments, the cells are COS cells, *e.g.*, COS-7 cells

(ATCC CRL 1651) or COS-1 cells (ATCC CRL 1650); HEK293 cells (ATCC CRL 1573); or *Xenopus* melanophores.

In particular embodiments, the HG20 protein comprises the amino acid sequence shown in SEQ.ID.NO.:2. In particular embodiments, the HG20 protein is a truncated HG20 protein. In particular embodiments, the truncated HG20 protein comprises amino acids 52-941 of SEQ.ID.NO.:2. In particular embodiments, the truncated HG20 protein is a chimeric HG20 protein.

The present invention includes HG20 protein substantially free from other proteins. The amino acid sequence of the full-length HG20 protein is shown in Figure 2 as SEQ.ID.NO.:2. Thus, the present invention includes polypeptides comprising HG20 protein substantially free from other proteins where the polypeptides comprise the amino acid sequence SEQ.ID.NO.:2. The present invention also includes polypeptides comprising HG20 proteins lacking a signal sequence. Examples of amino acid sequences of HG20 proteins lacking a signal sequence are:

Positions 9-941 of SEQ.ID.NO.:2;
Positions 35-941 of SEQ.ID.NO.:2;
Positions 36-941 of SEQ.ID.NO.:2;
Positions 38-941 of SEQ.ID.NO.:2;
Positions 39-941 of SEQ.ID.NO.:2;
Positions 42-941 of SEQ.ID.NO.:2;
Positions 44-941 of SEQ.ID.NO.:2;
Positions 46-941 of SEQ.ID.NO.:2;
Positions 52-941 of SEQ.ID.NO.:2; and
Positions 57-941 of SEQ.ID.NO.:2.

The present invention also includes DNA encoding the above-described HG20 proteins lacking a signal sequence. Thus, *e.g.*, the present invention includes a DNA molecule comprising a nucleotide sequence selected from the group consisting of:

Positions 293-3,115 of SEQ.ID.NO.:1;
Positions 317-3,115 of SEQ.ID.NO.:1;
Positions 395-3,115 of SEQ.ID.NO.:1;
Positions 398-3,115 of SEQ.ID.NO.:1;

Positions 404-3,115 of SEQ.ID.NO.:1;
Positions 407-3,115 of SEQ.ID.NO.:1;
Positions 416-3,115 of SEQ.ID.NO.:1;
Positions 422-3,115 of SEQ.ID.NO.:1;
Positions 428-3,115 of SEQ.ID.NO.:1;
Positions 446-3,115 of SEQ.ID.NO.:1; and
Positions 461-3,115 of SEQ.ID.NO.:1.

As with many receptor proteins, it is possible to modify many of the amino acids of HG20, particularly those which are not found in the ligand binding domain, and still retain substantially the same biological activity as the original protein. Thus this invention includes modified HG20 polypeptides which have amino acid deletions, additions, or substitutions but that still retain substantially the same biological activity as native HG20. It is generally accepted that single amino acid substitutions do not usually alter the biological activity of a protein (see, *e.g.*, Molecular Biology of the Gene, Watson et al., 1987, Fourth Ed., The Benjamin/Cummings Publishing Co., Inc., page 226; and Cunningham & Wells, 1989, Science 244:1081-1085). Accordingly, the present invention includes polypeptides where one amino acid substitution has been made in SEQ.ID.NO.:2 or in one of the HG20 polypeptides lacking a signal sequence listed above, wherein the polypeptides still retain substantially the same biological activity as native HG20. The present invention also includes polypeptides where two or more amino acid substitutions have been made in SEQ.ID.NO.:2 or in one of the HG20 polypeptides lacking a signal sequence listed above, wherein the polypeptides still retain substantially the same biological activity as native HG20. In particular, the present invention includes embodiments where the above-described substitutions are conservative substitutions. In particular, the present invention includes embodiments where the above-described substitutions do not occur in the ligand-binding domain of HG20. In particular, the present invention includes embodiments where amino acid changes have been made in the positions of HG20 where the amino acid sequence of HG20 differs from the amino acid sequence of $GABA_BR1b$ (see Figure 8).

The present invention also includes C-terminal truncated forms of HG20, particularly those which encompass the extracellular portion of the receptor, but lack the intracellular signaling portion of the receptor. Such truncated receptors are useful in various binding assays described herein, for crystallization studies, and for structure-activity-relationship studies. Accordingly, the present invention includes an HG20 protein substantially free from other proteins having the amino acid sequence of positions 1-480 of SEQ.ID.NO.:2.

O'Hara et al., 1993, Neuron 11:41-52 (O'Hara) reported that the amino terminal domains of several metabotropic glutamate receptors showed amino acid sequence similarities to the amino termini of several bacterial periplasmic binding proteins. O'Hara used this similarity to predict, and then experimentally confirm, that these amino terminal domains correspond to the location of the ligand binding domains of these metabotropic glutamate receptors.

The present inventors have discovered a region of amino acid sequence in the N-terminal domain of HG20 that is similar to the amino acid sequence of the bacterial periplasmic binding protein Leucine, Isoleucine, Valine (Alanine and Threonine) Binding Protein (LIVAT-BP) of *Pseudomonas aeruginosa*. See Figure 6. The region shown is about 25% identical between the two proteins. This is above the maximum identity of 17% reported by O'Hara between any one metabotropic glutamate receptor and any one periplasmic binding protein and indicates that the region of HG20 depicted is highly likely to contain the ligand binding domain.

Accordingly, the present invention includes a polypeptide representing the ligand binding domain of HG20 that includes amino acids 63-259 of SEQ.ID.NO.:2. Also provided are chimeric proteins comprising amino acids 63-259 of SEQ.ID.NO.:2.

Romano et al., 1996, J. Biol. Chem. 271:28612-28616 demonstrated that metabotropic glutamate receptors are often found as homodimers formed by an intermolecular disulfide bond. The location of the cysteines responsible for the disulfide bond was found to be in the amino terminal 17kD of the receptors. Transmembrane interactions may

also contribute to functional GABA$_B$ receptor dimer formation, as previously reported for the dopamine D2 receptor and β2-adrenergic receptor (Ng et al., 1996, Biochem. Biophys. Res. Comm. 227:200-204; Hebert et al., 1996, J. Biol. Chem. 271, 16384-16392). Accordingly, the present invention includes dimers of HG20 proteins. In particular embodiments, the HG20 protein has an amino acid selected from the group consisting of:

SEQ.ID.NO.:2;
Positions 9-941 of SEQ.ID.NO.:2;
Positions 35-941 of SEQ.ID.NO.:2;
Positions 36-941 of SEQ.ID.NO.:2;
Positions 38-941 of SEQ.ID.NO.:2;
Positions 39-941 of SEQ.ID.NO.:2;
Positions 42-941 of SEQ.ID.NO.:2;
Positions 44-941 of SEQ.ID.NO.:2;
Positions 46-941 of SEQ.ID.NO.:2;
Positions 52-941 of SEQ.ID.NO.:2;
Positions 57-941 of SEQ.ID.NO.:2; and
Positions 1-480 of SEQ.ID.NO.:2.

It has been found that, in some cases, membrane spanning regions of receptor proteins can be used to inhibit receptor function (Ng et al., 1996, Biochem. Biophys. Res. Comm. 227:200-204; Hebert et al., 1996, J. Biol. Chem. 271, 16384-16392; Lofts et al., Oncogene 8:2813-2820). Accordingly, the present invention provides peptides derived from the seven membrane spanning regions of HG20 and their use to inhibit HG20 or GABA$_B$ receptor function. Such peptides can include the whole or parts of the membrane spanning domains.

The present invention also includes isolated forms of HG20 proteins. By "isolated HG20 protein" is meant HG20 protein that has been isolated from a natural source or produced by recombinant means. Use of the term "isolated" indicates that HG20 protein is not present in its normal cellular environment. Thus, an isolated HG20 protein may be in a cell-free solution or placed in a different cellular environment from that in which it occurs naturally. The term isolated does not imply that an

isolated HG20 protein is the only protein present. but instead means that an isolated HG20 protein is at least 95% free of non-amino acid material (*e.g.*, nucleic acids, lipids, carbohydrates) naturally associated with the HG20 protein. Thus, an HG20 protein that is expressed through recombinant means in bacteria or even in eukaryotic cells which do not naturally (*i.e.*, without human intervention) express it is an "isolated HG20 protein."

The present invention also includes chimeric HG20 proteins. By chimeric HG20 protein is meant a contiguous polypeptide sequence of HG20 fused in frame to a polypeptide sequence of a non-HG20 protein. For example, the N-terminal domain and seven transmembrane spanning domains of HG20 fused at the C-terminus in frame to a G protein would be a chimeric HG20 protein. Another example of a chimeric HG20 protein would be a polypeptide comprising the FLAG epitope fused in frame at the amino terminus of amino acids 52-941 of SEQ.ID.NO.:2.

The present invention also includes HG20 proteins that are in the form of multimeric structures, *e.g.*, dimers. Such multimers of other metabotropic G-protein coupled receptors are known (Hebert et al., 1996, J. Biol. Chem. 271, 16384-16392; Ng et al., 1996, Biochem. Biophys. Res. Comm. 227, 200-204; Romano et al., 1996, J. Biol. Chem. 271, 28612-28616).

Preferred forms of dimers of HG20 are heterodimers comprising HG20 and other G-protein coupled receptors (GPCRs). Such GPCRs could be, *e.g.*, other subunits of $GABA_B$ receptors, proteins from *C. elegans* showing homology to HG20 (see Figure 24), or human GPCRs that are homologs of the *C. elegans* proteins. Particularly preferred forms of heterodimers are heterodimers of HG20 and either $GABA_BR1a$ or $GABA_BR1b$. It has been found by the present inventors that such heterodimers exhibit functional properties of $GABA_B$ receptors while monomers or homodimers of HG20, $GABA_BR1a$, or $GABA_BR1b$ do not exhibit functional properties. Another likely heterodimer partner for HG20 is the protein corresponding to the sequence deposited in GenBank at accession number 3776096.

The strongest evidence that functional GABA$_B$ receptors require both HG20 and GABA$_B$R1a or GABA$_B$R1b comes from studies demonstrating that co-transfection and co-expression of both HG20 and either GABA$_B$R1a or GABA$_B$R1b is necessary in order for the detection of GABA$_B$ receptor functional responses. Transfection and expression of HG20, GABA$_B$R1a, or GABA$_B$R1b alone does not lead to the production of functional GABA$_B$ receptors.

For example, in *Xenopus* melanophores co-expressing HG20 and GABA$_B$R1a, but not in melanophores expressing HG20 or GABA$_B$R1a alone, or in mock transfected melanophores, GABA mediated a dose-dependent pigment aggregation response that could be inhibited with the GABA$_B$ receptor specific CGP71872 antagonist. This pigment aggregation response is associated with a decrease in intracellular cAMP levels. Such a decrease has been confirmed in HEK293 cells. Also, co-expression of HG20 and GABA$_B$R1a in *Xenopus* oocytes resulted in the stimulation of inwardly rectifying potassium currents (Kirs). Native functional GABA$_B$ receptors have been reported to couple to Kirs (Misgeld et al., 1995, Prog. Neurobiol. 46:423-462).

Consistent with the need for both HG20 and GABA$_B$R1a for detection of functional GABA$_B$ receptors in transfected cells, the present inventors have demonstrated that HG20 and GABA$_B$R1a form heterodimers by immunoprecipitation of HG20 followed by immunoblotting with a GABA$_B$R1a antibody.

That a functional GABA$_B$ receptor requires both HG20 and either GABA$_B$R1a or GABA$_B$R1b is also suggested by the observation that GABA$_B$R1a or GABA$_B$R1b, recombinantly expressed in the absence of HG20, binds ligand with much reduced affinity compared to the affinity of native GABA$_B$ receptors. Also, characterization of the tissue distribution of each of the receptors by *in situ* hybridization histochemistry in rat brain revealed co-localization of HG20 and GABA$_B$R1a transcripts in many brain regions, including cortex, at both the regional and cellular levels.

The *Xenopus* melanophore pigment aggregation/dispersion assay has been shown to be highly suitable for monitoring agonist

activation of Gi-, Gq-, and Gs-coupled receptors (Potenza et al., 1992, Anal. Biochem. 206:315-322; Lerner, 1994, Trends Neurosci. 17:142-146). Agonist activation of Gi-coupled receptors expressed in melanophores results in pigment aggregation via a reduction in intracellular cAMP levels, whereas activation of Gs- and Gq-coupled receptors results in pigment dispersion via elevations in intracellular cAMP and calcium levels, respectively. Melanophores transfected separately with either $GABA_BR1a$ or HG20 showed no pigment aggregation or dispersion response following treatment with up to 1 mM concentrations of (L)-glutamic acid, GABA, or prototypic GABAergic agonists: (-)baclofen, 3-aminopropyl-(methyl)phosphonic acid, cis-4-aminocrotonic acid, piperidine-4-sulfonic acid (data not shown). Similarly, both receptors failed to couple to K^+ channels in *Xenopus* oocytes under patch-clamp conditions when transfected separately (data not shown). However, in melanophores transiently co-transfected with $GABA_BR1a$ and HG20, GABA mediated a dose-dependent aggregation response with an IC_{50} value of 3-7 μM (n=3). This aggregation was absent in mock-transfected cells and in cells transfected with $GABA_BR1a$ or HG20 alone (Figure 11). The GABA-mediated activity represented 42-56% (n=3) of a control cannabinoid receptor subtype 2 response (Figure 11, inset), and could be inhibited by the CGP71872 antagonist (n=3), indicating it was $GABA_B$ receptor specific (Figure 11). $GABA_BR1a$ was expressed by subcloning full-length $GABA_BR1a$ into the NheI-NotI site of pcDNA3.1 or pCIneo; HG20 was expressed as a FLAG-HG20 chimeric protein. See Examples 11 and 20 for further experimental details of expression vectors used, transfection conditions, assay conditions, *etc.* for the above-described co-expression studies.

The functional data arising from co-expression of $GABA_BR1a$ and HG20 receptors were confirmed in HEK293 cells. HEK293 cells transfected with and stably expressing $GABA_BR1a$ and HG20 were selected based on expression of receptor message as determined by dot blot analyses. In cell lines stably expressing the individual receptors, we observed small and inconsistent responses in assays to examine agonist-mediated modulation of cAMP synthesis. However, transient transfection

of HEK293 cells stably expressing GABA$_B$R1a (rgb1a-50) with an HG20 expression plasmid and transient transfection of HEK293 cells stably expressing HG20 (hgb2-42) with a GABA$_B$R1a expression plasmid significantly enhanced the ability of baclofen and GABA to inhibit forskolin-stimulated cAMP synthesis. Rgb1a-50 cells transfected with HG20 exhibited a 28% reduction in forskolin-stimulated cAMP synthesis with 30 μM baclofen and a 40% decrease with 30 μM GABA plus 100 μM aminooxyacetic acid (AOAA; a GABA transaminase inhibitor) and 100 μM nipecotic acid (a GABA uptake inhibitor) (Figure 12B). A 34% reduction in forskolin-stimulated cAMP synthesis was observed for hgb2-42 cells transfected with GABA$_B$R1a treated with baclofen and a 43% decrease was observed for GABA plus AOAA and nipecotic acid (Figure 12B). While inhibition of cAMP synthesis was sometimes observed with rgb1a-50 cells transfected with GABA$_B$R1a and hgb2-42 cells transfected with HG20, these effects were small and inconsistent (0-20% inhibition; Figure 12B). Neither baclofen nor GABA plus AOAA and nipecotic acid in the absence of forskolin had any affect on cAMP synthesis (Figure 12B). In addition, wild-type HEK293 cells did not exhibit baclofen- or GABA-mediated inhibition of forskolin-stimulated cAMP synthesis (Figure 12B). These data demonstrate that the functional GABA$_B$ receptor requires both GABA$_B$R1a and HG20. For experimental details of these studies in HEK293 cells, see Example 12.

Co-expression of the GABA$_B$R1a and HG20 with the inwardly rectifying potassium channels Kir 3.1/3.2 in *Xenopus* oocytes resulted in a significant stimulation of inwardly rectifying potassium current (Kir) in response to GABA [301 +/- 20.6 %, (n=3) increase over control current] measured at -80 mV which could subsequently be washed out with control solution (Figure 13). Modulation of Kir 3.1/3.2 was not seen in oocytes expressing GABA$_B$R1a or HG20 individually, even in the presence of Giα1 (Figure 13). See Example 21 for details.

To determine whether receptor intermolecular interactions accounted for the functional activity that was observed following the co-expression of recombinant GABA$_B$R1a and HG20, membranes from cells co-expressing GABA$_B$R1a and HG20 or the individual proteins were first

immunoprecipitated using anti-FLAG antibodies (to detect the recombinant FLAG-HG20 chimeric proteins) followed by immunoblotting with a $GABA_BR1a$-specific antibody. As seen in Figure 14, lanes 1-3, no $GABA_BR1a$ immunoreactivity was detected in samples prepared from mock vector transfected cells, FLAG-HG20 alone expressing cells, and $GABA_BR1a$ alone expressing cells immunoprecipitated with the FLAG-antibody. Since immunoreactive species were detected only in cells co-expressing HG20 and $GABA_BR1a$, this experiment demonstrates that HG20 and $GABA_BR1a$ can only be co-immunoprecipitated as part of a complex (Figure 14, lane 4). Based on the predicted molecular mass of a heterodimer of HG20 and $GABA_BR1a$, the ~250+ and ~130 kDa species may represent a heterodimer and $GABA_BR1a$ monomers, respectively. The stability of the HG20/$GABA_BR1a$ heterodimer in denaturing and reducing conditions suggests that SDS-stable transmembrane interactions form the heterodimer, as reported previously for β2 adrenergic and dopamine D2 receptors (Ng et al., 1996, Biochem. Biophys. Res. Comm. 227:200-204; Hebert et al., 1996, J. Biol. Chem. 271, 16384-16392). The monomer might result from partial disruption, subsequent to immunoprecipitation, of N-terminal Sushi repeats, C-terminal alpha-helical interacting domains (*e.g.*, coiled-coils) present in HG20 and $GABA_BR1a$ subunits, transmembrane interactions, or disulfide bonds that contribute to forming the heterodimer.

Particular examples of such regions likely to be involed in forming the heterodimer are shown in Figure 23. Regions such as those shown in Figure 23, as well as polypeptides comprising such regions are expected to be useful for the purpose of modulating the formation of heterodimers involving HG20 and thus controlling $GABA_B$ receptor activity. Accordingly, the present invention includes polypeptides comprising the coiled-coil domains of HG20, $GABA_BR1a$, and $GABA_BR1b$. In particular, the present invention includes polypeptides comprising an amino acid sequence selected from the group consisting of: positions 756-829 of SEQ.ID.NO.:2; positions 779-814 of SEQ.ID.NO.:2; positions 886-949 of SEQ.ID.NO.:21; and positions 889-934 of SEQ.ID.NO.:21; where the polypeptides do not contain other contiguous amino acid sequences

longer than 5 amino acids from a $GABA_B$ receptor subunit. The present invention also includes heterodimers of such polypeptides. In more general terms, the present invention includes comprising a coiled-coil domain from a first $GABA_B$ receptor subunit and no other contiguous amino acid sequences longer than 5 amino acids from the first $GABA_B$ receptor subunit where the coiled-coil domain is present in the C-terminus of the first $GABA_B$ receptor subunit and mediates heterodimerization of the first $GABA_B$ receptor subunit with a second $GABA_B$ receptor subunit.

In addition to the coiled-coil domains discussed above, a variety of regions of HG20 and $GABA_B$R1a are expected to be important for heterodimer formation. Motif analysis of the N-terminus of murine $GABA_B$R1a revealed seven consensus N-linked glycosylation sites and three putative short consensus repeats (SCRs) of ~60 amino acids each: amino acids 27-96 and amino acids 102-157 ($GABA_B$R1a specific), and amino acids 183-245 (common to $GABA_B$R1b (Kaupmann et al., 1997, Nature 386:239-246) and HG20 (Jones et al., 1998, Nature 396:674-679; White et al., 1998, Nature 396:679-682; Kaupmann et al., 1998, Nature 396:683-687; Kuner et al., 1999, Science 283:74-77) not described previously (Figure 26A-B). Since SCRs are known to play important roles in protein-protein interactions in a wide variety of complement proteins, adhesion proteins, and selectins (Chou and Heinrikson, 1997, J. Protein Chem. 16:765-773; Perkins et al., 1998, Biochemistry 27:4004-4012), of which the latter shows weak amino acid identity to murine $GABA_B$R1a, these SCRs, together with the coiled-coil domains discussed above in the carboxyl tails of $GABA_B$R1a and HG20 (Figure 23), are expected to be involved in the heterodimerization of $GABA_B$R1a and HG20.

Therefore, the present invention includes a polypeptide comprising an SCR domain from a first $GABA_B$ receptor subunit and no other contiguous amino acid sequences longer than 5 amino acids from the first $GABA_B$ receptor subunit where the SCR domain is present in the N-terminus of the first $GABA_B$ receptor subunit and mediates heterodimerization of the first $GABA_B$ receptor subunit with a second $GABA_B$ receptor subunit. In particular embodiments, the SCR is selected from the group consisting of: positions 27-96 of SEQ.ID.NO.:20; positions

102-157 of SEQ.ID.NO.:20; positions 183-245 of SEQ.ID.NO.:20; positions 28-97 of SEQ.ID.NO.:21; positions 103-158 of SEQ.ID.NO.:21; positions 184-246 of SEQ.ID.NO.:21; positions 4-22 of SEQ.ID.NO.:2; positions 23-49 of SEQ.ID.NO.:2; and positions 72-135 of SEQ.ID.NO.:2.

As in the metabotropic glutamate receptors (mGLURs), the second intracellular loop of murine GABABR1a is rich in basic amino acids which may play a role in G-protein-interactions (reviewed by Pin and Duvoisin, 1995, Neuropharmacology 34:1-26), and, as in the mGLURs, the carboxyl tail of murine GABABR1a contains a PDZ protein-interacting module (serine-arginine-valine, amino acids 953-955) which has been shown for mGLURs to play an important role in the interactions among the signaling components of synaptic junctions (Brakeman et al.1997, Nature 386:284-288). The murine GABABR1a receptor also contains potential protein kinase C and casein kinase II recognition sites predicted using ProSearch (Kolakowski et al., 1992, Biotechniques 13:919-921).

The present invention also relates to the identification and cloning of the murine GABABR1a receptor, the murine ortholog of the rat GABABR1a receptor described in Kaupmann et al., 1997, Nature 386:239-246 (Kaupmann). The present invention provides DNA encoding murine GABABR1a that is substantially free from other nucleic acids. The present invention also provides recombinant DNA molecules encoding murine GABABR1a.

The present invention provides a DNA molecule encoding murine GABABR1a that is substantially free from other nucleic acids and comprises the nucleotide sequence shown in Figure 15 as SEQ.ID.NO.:19. The open reading frame of SEQ.ID.NO.:19, encoding mouse GABABR1a protein, is positions 1-2,880, with positions 2,881-2,883 repesenting a stop codon. Thus, the present invention also provides a DNA molecule substantially free from other nucleic acids comprising the nucleotide sequence of positions 1-2,880 of SEQ.ID.NO.:19.

Sequence analysis of the open reading frame of the murine GABABR1a DNA revealed that it encodes a mature protein (*i.e.*, lacking a signal sequence) of 942 amino acids with a predicted molecular weight of

106.5 kDa that is 99% identical to rat GABA$_B$R1a (described in Kaupmann), with only six amino acid changes overall. Murine GABA$_B$R1a protein shares 31% overall amino acid identity to HG20.

CGP71872 is a photoaffinity ligand specific for GABA$_B$R1a receptors ($K_d = 1.0 \pm 0.2$ nM) that can be cross-linked to rat GABA$_B$R1a (Kaupmann et al., 1997, Nature 386:239-246). In crude membranes prepared from COS-7 cells transiently transfected with murine GABA$_B$R1a, [^{125}I]CGP71872 photolabelled a major band at ~130 kDa representing the mature (presunably glycosylated) protein and an additional band at approximately twice that molecular weight, possibly representing dimers (Figure 9). Ligand-binding species could also be detected with affinity purified GABA$_B$R1a antibodies 1713.1 (raised against the peptide acetyl-DVNSRRDILPDYELKLC-amide; a port ion of SEQ.ID.NO.:20) and 1713.2 (raised against the peptide acetyl-CATLHNPTRVKLFEK-amide; a portion of SEQ.ID.NO.:20) (Figure 9). In contrast, FLAG-tagged HG20 protein did not bind the high-affinity CGP71872 ligand, although expression of the protein was confirmed by immunoblot analysis (Figure 9).

Displacement of [^{125}I]CGP71872 binding to recombinant murine GABA$_B$R1a was in the appropriate rank order of potency for GABAergic ligands: CGP71872 > SKF-97541 (3-aminopropyl(methyl)-phosphinic acid) > GABA > (-)baclofen > saclofen > (L)-glutamic acid.. Interestingly, recombinant rat GABA$_B$R1a exhibits 10-25 fold lower affinity for agonists than native GABA$_B$ receptors in brain (Kaupmann et al., 1997, Nature 386:239). Although the reason for this discrepancy remains unclear, a recent report indicated that recombinant GABA$_B$R1a may require additional cellular components for functional targeting to the plasma membrane (Couve et al., 1998, J. Biol. Chem. 273:26361-26367). Thus, GABA$_B$R1a alone, without such additional components, might be expected to exhibit somewhat altered ligand binding characteristics.

In the binding experiments discussed above using GABA$_B$R1a alone, surprisingly, dose-dependent displacement was not detected for (+)baclofen, and the affinities of agonists (GABA, SKF-97541, and (-)baclofen) and partial agonists ((+)baclofen, saclofen, (L)-glutamic

acid) but not the affinity of antagonist (CGP71872) for the recombinant $GABA_BR1a$ were markedly lower compared to native receptors in rat brain (Table 1). Agonist affinities of co-expressed HG20 and $GABA_BR1a$ were examined in membranes prepared from cells co-expressing $GABA_BR1a$ and FLAG-tagged HG20. Competition of [^{125}I]CGP71872 binding in these membranes showed recovery of high-affinity ligand binding comparable to native receptors in rat brain (Table 1). The simplest explanation for these results is that the high-affinity agonist binding pocket may comprise interactions between the N-terminal domains of HG20 and $GABA_BR1a$ that form the heterodimer.

Table 1

Ligand	rat cortex*	gb1a	gb1a/gb2
CGP71872	0.5 nM	0.52 - 0.67 nM	0.15 - 0.27 nM
GABA	2.5 uM	42.55 - 68.38 uM	1.77 - 2.55 uM
SKF-97541**	not determined	11.09 - 11.47 uM	0.80 - 0.96 uM
(-)Baclofen	0.5 uM	31.46 - 53.70 uM	3.92 - 7.78 uM
(+)Baclofen	not determined	no fit	1.25 - 3.94 mM
Saclofen	156 uM	280.5 - 365.0 uM	119.4 - 131.4 uM
L-Glutamate	not determined	119.4 - 285.0 mM	116.2 - 201.6 mM

* reported by Kaupmann et al., (1997) *Nature* 386, 239-246
** 3-aminopropyl(methyl)phosphinic acid

In Table 1, gb1a refers to $GABA_BR1a$ and gb1a/gb2 refers to HG20/ $GABA_BR1a$ heterodimers.

Co-localization studies were performed to determine if mRNAs for $GABA_BR1a$ and HG20 co-exist in the same cells in the brain. Figure 10A-B shows equivalent levels of $GABA_BR1a$ and HG20 hybridization in adjacent coronal sections of rat parietal cortex, indicating that messages for both receptors are expressed in this brain region. Radiolabelled and fluorescent probes for the two receptors were used to look at the cellular level where it was observed that message for both receptors is expressed in the same cells (Example 13 and Figure 10C-E). In the parietal cortex and all other major brain regions studied, including the hippocampus, thalamus, cerebellum, and vestibular ganglion, $GABA_BR1a$ and HG20 mRNAs are co-localized in the same cells. These results suggest that the functional native $GABA_B$ receptors found in these

cells involve both $GABA_BR1a$ and HG20. Co-immunoprecipitation, functional, and anatomical data described herein converge to strongly support the argument that the native, functional $GABA_B$ receptor is a heterodimer of $GABA_BR1a$ and HG20. This work is particularly exciting because it represents the first example of a heteromeric G protein-coupled receptor.

The novel murine $GABA_BR1a$ DNA sequences of the present, in whole or in part, can be linked with other DNA sequences, *i.e.*, DNA sequences to which $GABA_BR1a$ DNA is not naturally linked, to form "recombinant DNA molecules" encoding murine $GABA_BR1a$. Such other sequences can include DNA sequences that control transcription or translation such as, *e.g.*, translation initiation sequences, promoters for RNA polymerase II, transcription or translation termination sequences, enhancer sequences, sequences that control replication in microorganisms, or that confer antibiotic resistance. The novel DNA sequences of the present invention can be inserted into vectors such as plasmids, cosmids, viral vectors, or yeast artificial chromosomes.

The present invention also includes isolated forms of DNA encoding $GABA_BR1a$. By "isolated DNA encoding $GABA_BR1a$" is meant DNA encoding $GABA_BR1a$ that has been isolated from a natural source or produced by recombinant means. Use of the term "isolated" indicates that DNA encoding $GABA_BR1a$ is not present in its normal cellular environment. Thus, an isolated DNA encoding $GABA_BR1a$ may be in a cell-free solution or placed in a different cellular environment from that in which it occurs naturally. The term isolated does not imply that isolated DNA encoding $GABA_BR1a$ is the only DNA present. but instead means that isolated DNA encoding $GABA_BR1a$ is at least 95% free of non-nucleic acid material (*e.g.*, proteins, lipids, carbohydrates) naturally associated with the DNA encoding $GABA_BR1a$. Thus, DNA encoding $GABA_BR1a$ that is expressed in bacteria or even in eukaryotic cells which do not naturally (*i.e.*, without human intervention) contain it through recombinant means is "isolated DNA encoding $GABA_BR1a$."

Another aspect of the present invention includes host cells that have been engineered to contain and/or express DNA sequences

encoding murine GABA$_B$R1a. Such recombinant host cells can be cultured under suitable conditions to produce murine GABA$_B$R1a protein. An expression vector containing DNA encoding the murine GABA$_B$R1a protein can be used for expression of the murine GABA$_B$R1a protein in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to, bacteria such as *E. coli*, fungal cells such as yeast, mammalian cells including, but not limited to, cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to *Drosophila* and silkworm derived cell lines. Cell lines derived from mammalian species which are suitable for recombinant expression of the murine GABA$_B$R1a protein and which are commercially available, include but are not limited to, L cells L-M(TK$^-$) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), HEK293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), *Xenopus* melanophores, and *Xenopus* oocytes.

A variety of mammalian expression vectors can be used to express recombinant murine GABA$_B$R1a in mammalian cells. Commercially available mammalian expression vectors which are suitable include, but are not limited to, pMC1neo (Stratagene), pSG5 (Stratagene), pcDNAI and pcDNAIamp, pcDNA3, pcDNA3.1, pCR3.1 (Invitrogen), EBO-pSV2-neo (ATCC 37593), pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), and the PT7TS oocyte expression vector (or similar expression vectors containing the globin 5' UTR and the globin 3' UTR). Following expression in recombinant cells, the murine GABA$_B$R1a protein can be purified by conventional techniques to a level that is substantially free from other proteins.

Other cells that are particularly suitable for expression of the murine GABA$_B$R1a protein are immortalized melanophore pigment cells from *Xenopus laevis*. Such melanophore pigment cells can be used for functional assays using recombinant expression of murine GABA$_B$R1a in

a manner similar to the use of such melanophore pigment cells for the functional assay of other recombinant GPCRs (Graminski et al., 1993, J. Biol. Chem. 268:5957-5964; Lerner, 1994, Trends Neurosci. 17:142-146; Potenza & Lerner, 1992, Pigment Cell Res. 5:372-378; Potenza et al., 1992, Anal. Biochem. 206:315-322).

The present invention includes a method of producing the murine $GABA_BR1a$ protein comprising:

(a) transfecting a host cell with a expression vector comprising DNA that encodes the murine $GABA_BR1a$ protein;

(b) growing the host cells under conditions such that the murine $GABA_BR1a$ protein is produced; and

(c) recovering the murine $GABA_BR1a$ protein from the host cells.

In particular embodiments, the method of recovering the murine $GABA_BR1a$ protein may involve obtaining membrane preparations from the host cells that contain the murine $GABA_BR1a$ protein. Such membrane preparations may contain heterodimers of $GABA_BR1a$ protein and HG20 protein that form functional $GABA_B$ receptors.

In particular embodiments, the cells are eukaryotic cells. In other embodiments, the cells are mammalian cells. In still other embodiments, the cells are COS cells, in particular COS-7 cells (ATCC CRL 1651), COS-1 cells (ATCC CRL 1650), HEK293 cells (ATCC CRL 1573), or *Xenopus* melanophores.

The present inventors have discovered that, when either HG20 or $GABA_BR1a$ subunits are recombinantly expressed separately, *i.e.*, in different cells, very little or no expression is observed. It is only when HG20 and $GABA_BR1a$ subunits are recombinantly co-expressed, *i.e.*, expressed in the same cells at the same time, that high level expression of HG20 and $GABA_BR1a$ is observed (see Figure 25). Given the close relationship among $GABA_BR1a$, $GABA_BR1b$, *C. elegans* genes related to $GABA_BR1a$ and HG20 (see Figure 24), and the close relationship that is expected to be found between other isoforms of $GABA_BR1a$ and $GABA_BR1b$, it is believed that co-expression of HG20

and either GABABR1a, GABABR1b, *C. elegans* genes related to GABABR1a and HG20, or other isoforms of GABABR1a and GABABR1b will also result in increased expression of HG20 and GABABR1a, GABABR1b, *C. elegans* genes related to GABABR1a and HG20, or other isoforms of GABABR1a and GABABR1b as compared to expression of these proteins separately.

Accordingly, the present invention includes a method of co-expressing HG20 and GABABR1a, GABABR1b, *C. elegans* genes related to GABABR1a and HG20, or other isoforms of GABABR1a and GABABR1b so as to result in an increase in expression of HG20 and GABABR1a, GABABR1b, *C. elegans* genes related to GABABR1a and HG20, or other isoforms of GABABR1a and GABABR1b as compared to expression when HG20 and GABABR1a, GABABR1b, *C. elegans* genes related to GABABR1a and HG20, or other isoforms of GABABR1a and GABABR1b are expressed separately. In particular embodiments, the level of expression of HG20, GABABR1a, GABABR1b, *C. elegans* genes related to GABABR1a and HG20, or other isoforms of GABABR1a and GABABR1b is measured in the co-expressing cells. In particular embodiments, the level of expression of HG20, GABABR1a, GABABR1b, *C. elegans* genes related to GABABR1a and HG20, or other isoforms of GABABR1a and GABABR1b is measured by immunoblot or by immunoprecipitation/immunoblotting methods.

Thus, the present invention includes a method of increasing expression of HG20 and GABABR1a, GABABR1b, *C. elegans* genes related to GABABR1a and HG20, or other isoforms of GABABR1a and GABABR1b comprising:

(a) recombinantly expressing HG20 and GABABR1a, GABABR1b, *C. elegans* genes related to GABABR1a and HG20, or other isoforms of GABABR1a and GABABR1b in the same cells;

(b) measuring the expression of HG20, GABABR1a, GABABR1b, *C. elegans* genes related to GABABR1a and HG20, or other isoforms of GABABR1a and GABABR1b, where a measurement of detectable expression of HG20, GABABR1a, GABABR1b, *C. elegans* genes

related to $GABA_BR1a$ and HG20, or other isoforms of $GABA_BR1a$ and $GABA_BR1b$ indicates that increased expression has been achieved.

In particular embodiments, the measurement of expression is carried out by immunoblotting with or without immunoprecipitation.

In other embodiments, the method also comprises the steps of recombinantly expressing HG20 and $GABA_BR1a$, $GABA_BR1b$, *C. elegans* genes related to $GABA_BR1a$ and HG20, or other isoforms of $GABA_BR1a$ and $GABA_BR1b$ separately, measuring the level of expression of HG20, $GABA_BR1a$, $GABA_BR1b$, *C. elegans* genes related to $GABA_BR1a$ and HG20, or other isoforms of $GABA_BR1a$ and $GABA_BR1b$ in the separately expressing cells, and comparing the amount of expression of HG20, $GABA_BR1a$, $GABA_BR1b$, *C. elegans* genes related to $GABA_BR1a$ and HG20, or other isoforms of $GABA_BR1a$ and $GABA_BR1b$ in the separetely expressing cells to the amount of expression of HG20, $GABA_BR1a$, $GABA_BR1b$, *C. elegans* genes related to $GABA_BR1a$ and HG20, or other isoforms of $GABA_BR1a$ and $GABA_BR1b$ in the co-expressing cells.

Accordingly, the present invention includes a a method of increasing expression of HG20 and $GABA_BR1a$, $GABA_BR1b$, *C. elegans* genes related to $GABA_BR1a$ and HG20, or other isoforms of $GABA_BR1a$ and $GABA_BR1b$ comprising:

(a) recombinantly expressing HG20 and $GABA_BR1a$, $GABA_BR1b$, *C. elegans* genes related to $GABA_BR1a$ and HG20, or other isoforms of $GABA_BR1a$ and $GABA_BR1b$ in the same cells to form co-expressing cells;

(b) recombinantly expressing HG20 and $GABA_BR1a$, $GABA_BR1b$, *C. elegans* genes related to $GABA_BR1a$ and HG20, or other isoforms of $GABA_BR1a$ and $GABA_BR1b$ in different cells to form separately expressing cells;

(c) measuring the expression of HG20, $GABA_BR1a$, $GABA_BR1b$, *C. elegans* genes related to $GABA_BR1a$ and HG20, or other isoforms of $GABA_BR1a$ and $GABA_BR1b$ in the co-expressing cells;

(d) measuring the expression of HG20, $GABA_BR1a$, $GABA_BR1b$, *C. elegans* genes related to $GABA_BR1a$ and HG20, or other

isoforms of GABABR1a and GABABR1b in the separately expressing cells;

where if the amount of expression of HG20, GABABR1a, GABABR1b, *C. elegans* genes related to GABABR1a and HG20, or other isoforms of GABABR1a and GABABR1b is greater in the co-expressing cells as compared to the separately expressing cells, this indicates that increased expression has been achieved.

In particular embodiments, the measurement of expression is carried out by immunoblotting with or without immunoprecipitation.

The present invention includes murine GABABR1a protein substantially free from other proteins. The amino acid sequence of the full-length murine GABABR1a protein is shown in Figure 16 as SEQ.ID.NO.:20. Thus, the present invention includes polypeptides comprising the murine GABABR1a protein substantially free from other proteins having the amino acid sequence SEQ.ID.NO.:20. The present invention also includes murine GABABR1a protein lacking a signal sequence as well as DNA encoding such a protein. Such a murine GABABR1a protein lacking a signal sequence is represented by amino acids 18-960 of SEQ.ID.NO.:20.

The present invention includes modified murine GABABR1a polypeptides which have amino acid deletions, additions, or substitutions but that still retain substantially the same biological activity as native murine GABABR1a protein. The present invention includes polypeptides where one amino acid substitution has been made in SEQ.ID.NO.:20 or in a polypeptide represented by SEQ.ID.NO.:20 lacking a signal sequence, wherein the polypeptides still retain substantially the same biological activity as native murine GABABR1a protein. The present invention also includes polypeptides where two or more amino acid substitutions have been made in SEQ.ID.NO.:20 or in a polypeptide represented by SEQ.ID.NO.:20 lacking a signal sequence, wherein the polypeptides still retain substantially the same biological activity as native murine GABABR1a protein. In particular, the present invention includes embodiments where the above-described substitutions are conservative substitutions. In particular, the present invention includes embodiments

where the above-described substitutions do not occur in the ligand-binding domain of native murine $GABA_BR1a$ protein. In particular, the present invention includes embodiments where amino acid changes have been made in positions of native murine $GABA_BR1a$ protein where the amino acid sequence of native murine $GABA_BR1a$ protein differs from the amino acid sequence of HG20 when the amino acid sequences of native murine $GABA_BR1a$ protein and HG20 are aligned in a manner similar to the alignment of the amino acid sequences of $GABA_BR1b$ protein and HG20 shown in Figure 8.

The present invention also includes isolated forms of murine $GABA_BR1a$ proteins. By "isolated murine $GABA_BR1a$ protein" is meant murine $GABA_BR1a$ protein that has been isolated from a natural source or produced by recombinant means. Use of the term "isolated" indicates that murine $GABA_BR1a$ protein is not present in its normal cellular environment. Thus, an isolated murine $GABA_BR1a$ protein may be in a cell-free solution or placed in a different cellular environment from that in which it occurs naturally. The term isolated does not imply that an isolated murine $GABA_BR1a$ protein is the only protein present. but instead means that an isolated murine $GABA_BR1a$ protein is at least 95% free of non-amino acid material (*e.g.*, nucleic acids, lipids, carbohydrates) naturally associated with the murine $GABA_BR1a$ protein. Thus, an murine $GABA_BR1a$ protein that is expressed in bacteria or even in eukaryotic cells which do not naturally (*i.e.*, without human intervention) express it through recombinant means is an "isolated murine $GABA_BR1a$ protein."

The present invention also provides ligand-binding domains of murine $GABA_BR1a$ protein. A FASTA search of the database GenBank (bacterial division) using the N-terminal domain of murine $GABA_BR1a$ (amino acid positions 147-551 of SEQ.ID.NO.:20) as the probe reveals a match with the *E.coli* leucine-specific binding protein (livK) (22% identity over 339 amino acids), whereas no match to any bacterial amino acid binding protein is found using the receptor sequence inclusive of the region that includes the seven transmembrane domains (TM 1-7; amino acid positions 552-960) as a probe. The ligand-binding domain(s) of

$GABA_BR1a$ was also experimentally determined. Photoaffinity [125I]CGP71872 labeling experiments provided direct physical evidence that the N-terminal extracellular domain but not a C-terminal fragment of $GABA_BR1a$ (comprising TM1-7 and inclusive to the carboxyl tail) is responsible for ligand-binding (see Examples 14-19 and Figure 17B).

Accordingly, the present invention includes a polypeptide comprising the ligand binding domain of murine $GABA_BR1a$. In preferred embodiments, the polypeptide comprises amino acids 147-551 of SEQ.ID.NO.:20.

The present invention includes methods of identifying compounds that specifically bind to the $GABA_B$ receptor, as well as compounds identified by such methods. The specificity of binding of compounds showing affinity for the $GABA_B$ receptor is shown by measuring the affinity of the compounds for recombinant cells expressing HG20 and either $GABA_BR1a$ or $GABA_BR1b$, or for membranes from such cells. Expression of the $GABA_B$ receptor and screening for compounds that bind to the $GABA_B$ receptor or that inhibit the binding of a known, radiolabeled ligand of the $GABA_B$ receptor, *e.g.*, an amino acid or a GABA analogue such as (-)baclofen, to these cells, or membranes prepared from these cells, provides an effective method for the rapid selection of compounds with high affinity for the $GABA_B$ receptor. Other radiolabeled ligands that might be used are ibotenic acid, the amino acids glutamate and glycine, other amino acids, decarboxylated amino acids, or any of the other $GABA_B$ receptor ligands disclosed herein or known in the art. Such ligands need not necessarily be radiolabeled but can also be nonisotopic compounds that can be used to displace bound radiolabeled compounds or that can be used as activators in functional assays. Compounds identified by the methods disclosed herein are likely to be agonists or antagonists of the $GABA_B$ receptor and may be peptides, proteins, or non-proteinaceous organic molecules.

Therefore, the present invention includes assays by which $GABA_B$ receptor agonists and antagonists can be identified. Methods for identifying agonists and antagonists of other receptors are well known in the art and can often be adapted to identify agonists and antagonists of

the $GABA_B$ receptor. Accordingly, the present invention includes a method for determining whether a substance binds $GABA_B$ receptors and is thus a potential agonist or antagonist of the $GABA_B$ receptor that comprises:

(a) providing cells comprising an expression vector encoding HG20 and an expression vector encoding $GABA_BR1a$ or $GABA_BR1b$;

(b) culturing the cells under conditions such that HG20 and $GABA_BR1a$ or $GABA_BR1b$ are expressed and heterodimers of HG20 and $GABA_BR1a$ or $GABA_BR1b$ are formed;

(c) exposing the cells to a labeled ligand of $GABA_B$ receptors in the presence and in the absence of the substance;

(d) measuring the binding of the labeled ligand to the heterodimers of HG20 and $GABA_BR1a$ or $GABA_BR1b$ in the presence and in the absence of the substance;

where if the amount of binding of the labeled ligand is less in the presence of the substance than in the absence of the substance, then the substance is a potential agonist or antagonist of $GABA_B$ receptors.

Examples of ligands of $GABA_B$ receptors are: CGP71872, GABA, saclofen, (-)baclofen, glycine, and (L)-glutamic acid.

The present invention also includes a method for determining whether a substance is capable of binding to $GABA_B$ receptors, *i.e.*, whether the substance is a potential agonist or an antagonist of $GABA_B$ receptors, where the method comprises:

(a) providing test cells comprising an expression vector encoding HG20 and an expression vector encoding $GABA_BR1a$ or $GABA_BR1b$;

(b) culturing the test cells under conditions such that HG20 and $GABA_BR1a$ or $GABA_BR1b$ are expressed and heterodimers of HG20 and $GABA_BR1a$ or $GABA_BR1b$ are formed;

(c) exposing the test cells to the substance;

(d) measuring the amount of binding of the substance to the test cells;

(e) measuring the amount of binding of the substance to control cells;

(f) comparing the amount of binding of the substance to the test cells with the amount of binding of the substance to control cells;

where if the amount of binding of the substance to the test cells is greater than the amount of binding of the substance to control cells, then the substance is capable of binding to $GABA_B$ receptors;

where the control cells are essentially the same as the test cells except that the control cells do not comprise an expression vector encoding HG20 and an expression vector encoding $GABA_BR1a$ or $GABA_BR1b$.

Once a substance has been identified by the above-described methods, determining whether the substance is an agonist or antagonist can then be accomplished by the use of functional assays such as those described herein.

In particular embodiments, the cells are transfected with an expression vector encoding HG20 and an expression vector encoding $GABA_BR1a$ or $GABA_BR1b$.

In particular embodiments, the binding affinity of the substance for the test cells is determined. In particular embodiments, such binding affinity is between 1nM and 200 mM; preferably between 5 nM and 1 mM; more preferably between 10 nM and 100 μM; and even more preferably between 10 nM and 100 nM.

The conditions under which step (c) of the above-described methods is practiced are conditions that are typically used in the art for the study of protein-ligand interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

In a particular embodiment of the above-described methods, the cells are eukaryotic cells. In another embodiment, the cells are mammalian cells. In other embodiments, the cells are L cells L-M(TK^-) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), HEK293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC

CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), or *Xenopus* melanophores.

The assays described above can be carried out with cells that have been transiently or stably transfected with an expression vector encoding HG20 and an expression vector encoding $GABA_BR1a$ or $GABA_BR1b$. Transfection is meant to include any method known in the art for introducing HG20 and $GABA_BR1a$ or $GABA_BR1b$ into the test cells. For example, transfection includes calcium phosphate or calcium chloride mediated transfection, lipofection, infection with a retroviral construct, and electroporation. In particular embodiments, a single expression vector encodes HG20 and $GABA_BR1a$ or $GABA_BR1b$.

Where binding of the substance or ligand is measured, such binding can be measured by employing a labeled substance or ligand. The substance or ligand can be labeled in any convenient manner known to the art, *e.g.*, radioactively, fluorescently, enzymatically.

In particular embodiments of the above-described methods, the substance or ligand is an amino acid or an amino acid analogue such as CGP71872, GABA, saclofen, (-)baclofen, glycine, and (L)-glutamic acid.

In particular embodiments of the above-described methods, HG20 has an amino acid sequence of SEQ.ID.NO.:2.

In particular embodiments of the above-described methods, HG20 comprises an amino acid sequence selected from the group consisting of:

SEQ.ID.NO.:2;
Positions 9-941 of SEQ.ID.NO.:2;
Positions 35-941 of SEQ.ID.NO.:2;
Positions 36-941 of SEQ.ID.NO.:2;
Positions 38-941 of SEQ.ID.NO.:2;
Positions 39-941 of SEQ.ID.NO.:2;
Positions 42-941 of SEQ.ID.NO.:2;
Positions 44-941 of SEQ.ID.NO.:2;
Positions 46-941 of SEQ.ID.NO.:2;
Positions 52-941 of SEQ.ID.NO.:2; and

Positions 57-941 of SEQ.ID.NO.:2.

In particular embodiments, $GABA_BR1a$ is murine $GABA_BR1a$ and has the amino acid sequence SEQ.ID.NO.:20. In particular embodiments, $GABA_BR1a$ is rat $GABA_BR1a$ and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In particular embodiments, $GABA_BR1b$ is rat $GABA_BR1b$ and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In particular embodiments, $GABA_BR1a$ is human $GABA_BR1a$ and has an amino acid sequence selected from the group consisting of: SEQ.ID.NO.:21 and the protein encoded by SEQ.ID.NO.:23.

The above-described methods can be modified in that, rather than exposing cells to the substance, membranes can be prepared from the cells and those membranes can be exposed to the substance. Such a modification utilizing membranes rather than cells is well known in the art with respect to other receptors and is described in, *e.g.*, Hess et al., 1992, Biochem. Biophys. Res. Comm. 184:260-268.

As a further modification of the above-described method, RNA encoding HG20 and $GABA_BR1a$ or $GABA_BR1b$ can be prepared as, *e.g.*, by *in vitro* transcription using a plasmid containing HG20 and a plasmid containing $GABA_BR1a$ or $GABA_BR1b$ under the control of a bacteriophage T7 promoter, and the RNA can be microinjected into *Xenopus* oocytes in order to cause the expression of HG20 and $GABA_BR1a$ or $GABA_BR1b$ in the oocytes. Substances are then tested for binding to the heterodimer of HG20 and $GABA_BR1a$ or $GABA_BR1b$ expressed in the oocytes. Alternatively, rather than detecting binding, the effect of the substances on the electrophysiological properties of the oocytes can be determined.

The present invention includes assays by which $GABA_B$ receptor agonists and antagonists may be identified by their ability to stimulate or antagonize a functional response mediated by the $GABA_B$ receptor in cells that have been co-transfected with and that co-express HG20 and $GABA_BR1a$ or $GABA_BR1b$.

Accordingly, the present invention provides a method of identifying agonists and antagonists of HG20 comprising:

(a) providing test cells by transfecting cells with:

(1) an expression vector that directs the expression of HG20 in the cells; and

(2) an expression vector that directs the expression of $GABA_BR1a$ or $GABA_BR1b$ in the cells;

(b) exposing the test cells to a substance that is suspected of being an agonist of the $GABA_B$ receptor;

(c) measuring the amount of a functional response of the test cells that have been exposed to the substance;

(d) comparing the amount of the functional response exhibited by the test cells with the amount of the functional response exhibited by control cells;

wherein if the amount of the functional response exhibited by the test cells differs from the amount of the functional response exhibited by the control cells, the substance is an agonist or antagonist of the $GABA_B$ receptor;

where the control cells are cells that have not been transfected with HG20 and $GABA_BR1a$ or $GABA_BR1b$ but have been exposed to the substance or are test cells that have not been exposed to the substance.

In particular embodiments of the above-described methods, HG20 has an amino acid sequence of SEQ.ID.NO.:2.

In particular embodiments of the above-described methods, HG20 comprises an amino acid sequence selected from the group consisting of:

SEQ.ID.NO.:2;
Positions 9-941 of SEQ.ID.NO.:2;
Positions 35-941 of SEQ.ID.NO.:2;
Positions 36-941 of SEQ.ID.NO.:2;
Positions 38-941 of SEQ.ID.NO.:2;
Positions 39-941 of SEQ.ID.NO.:2;
Positions 42-941 of SEQ.ID.NO.:2;
Positions 44-941 of SEQ.ID.NO.:2;
Positions 46-941 of SEQ.ID.NO.:2;

Positions 52-941 of SEQ.ID.NO.:2; and

Positions 57-941 of SEQ.ID.NO.:2.

In particular embodiments, GABABR1a is murine GABABR1a and has the amino acid sequence SEQ.ID.NO.:20. In particular embodiments, GABABR1a is rat GABABR1a and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In particular embodiments, GABABR1b is rat GABABR1b and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In particular embodiments, GABABR1a is human GABABR1a and has an amino acid sequence selected from the group consisting of: SEQ.ID.NO.:21 and the protein encoded by SEQ.ID.NO.:23.

In particular embodiments, the functional response is selected from the group consisting of: changes in pigment distribution in melanophore cells; changes in cAMP or calcium concentration; and changes in membrane currents in *Xenopus* oocytes. In particular embodiments, the change in pigment distribution is pigment aggregation; the change in cAMP concentration is a decrease in cAMP concentration; the change in membrane current is the modulation of an inwardly rectifying potassium current.

In a particular embodiment of the above-described method, the cells are eukaryotic cells. In another embodiment, the cells are mammalian cells. In other embodiments, the cells are L cells L-M(TK$^-$) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), *Xenopus* melanophores, or *Xenopus* oocytes.

In a particular embodiment of the above-described method, the cells are transfected with separate expression vectors that direct the expression of HG20 and either GABABR1a or GABABR1b in the cells. In other embodiments, the cells are transfected with a single expression vector that direct the expression of both HG20 and GABABR1a or GABABR1b in the cells.

In a particular embodiment, the cells are *Xenopus* melanophores and the functional response is pigment aggregation. In another embodiment, the cells are HEK293 cells and the functional response is a decrease in cAMP level. In another embodiment, the cells are *Xenopus* oocytes and the functional response is the production of an inwardly rectifying potassium current.

The conditions under which step (b) of the method is practiced are conditions that are typically used in the art for the study of protein-ligand interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

The above-described assay can be easily modified to form a method to identify antagonists of the $GABA_B$ receptor. Such a method comprises:

(a) providing cells by transfecting cells with:

(1) an expression vector that directs the expression of HG20 in the cells; and

(2) an expression vector that directs the expression of $GABA_BR1a$ or $GABA_BR1b$ in the cells;

(b) exposing the cells to a substance that is a known agonist of the $GABA_B$ receptor;

(c) measuring the amount of a functional response of the cells that have been exposed to the known agonist;

(d) exposing the cells concurrently to the known agonist and to a substance that is suspected of being an antagonist of the $GABA_B$ receptor;

(e) measuring the amount of a functional response of the cells that have been exposed to the substance and the known agonist;

(f) comparing the amount of the functional response measured in step (c) with the amount of the functional response measured in step (e);

wherein if the amount of the functional response measured in step (c) is greater than the amount of the functional response measured in step (e), the substance is an antagonist of the $GABA_B$ receptor.

Additional types of functional assays that can be used to identify agonists and antagonists of $GABA_B$ receptors include transcription-based assays. Transcription-based assays involve the use of a reporter gene whose transcription is driven by an inducible promoter whose activity is regulated by a particular intracellular event such as, *e.g.*, changes in intracellular calcium levels that are caused by the interaction of a receptor with a ligand. Transciption-based assays are reviewed in Rutter et al., 1998, Chemistry & Biology 5:R285-R290.

The transcription-based assays of the present invention rely on the expression of reporter genes whose transcription is activated or repressed as a result of intracellular events that are caused by the interaction of an agonist with a heterodimer of HG20 and either $GABA_BR1a$ or $GABA_BR1b$ where the heterodimer forms a functional $GABA_B$ receptor.

An extremely sensitive transcription based assay is disclosed in Zlokarnik et al., 1998, Science 279:84-88 (Zlokarnik) and also in U.S. Patent No. 5,741,657. The assay disclosed in Zlokarnik and U.S. Patent No. 5,741,657 employs a plasmid encoding β-lactamase under the control of an inducible promoter. This plasmid is transfected into cells together with a plasmid encoding a receptor for which it is desired to identify agonists. The inducible promoter on the β-lactamase is chosen so that it responds to at least one intracellular signal that is generated when an agonist binds to the receptor. Thus, following such binding of agonist to receptor, the level of β-lactamase in the transfected cells increases. This increase in β-lactamase is made measurable by treating the cells with a cell-permeable dye that is a substrate for β-lactamase. The dye contains two fluorescent moieties. In the intact dye, the two fluorescent moieties are close enough to one another that fluorescent resonance energy transfer (FRET) can take place between them. Following cleavage of the dye into two parts by β-lactamase, the two fluorescent moitites are located on different parts, and thus can drift apart. This increases the distance betweeen the flourescent moities, thus decreasing the amount of FRET that can occur between them. It is this decrease in FRET that is measured in the assay.

One skilled in the art can modify the assay described in Zlokarnik and U.S. Patent No. 5,741,657 to form an assay for identifying agonists of $GABA_B$ receptors by using an inducible promoter to drive β-lactamase that is activated by an intracellular signal generated by the interaction of agonists and the $GABA_B$ receptor. To produce the $GABA_B$ receptor, a plasmid encoding HG20 and a plasmid encoding $GABA_BR1a$ or $GABA_BR1b$ would be transfected into the cells. The cells would be exposed to the cell-permeable dye and then exposed to substances suspected of being agonists of the $GABA_B$ receptor. Those substances that cause a decrease in FRET are likely to actually be agonists of the $GABA_B$ receptor.

Accordingly, the present invention includes a method for identifying agonists of the $GABA_B$ receptor comprising:

(a) transfecting cells with:

(1) an expression vector that directs the expression of HG20 in the cells;

(2) an expression vector that directs the expression of $GABA_BR1a$ or $GABA_BR1b$ in the cells;

(3) an expression vector that directs the expression of β-lactamase under the control of an inducible promoter that is activated by an intracellular signal generated by the interaction of agonists and the $GABA_B$ receptor;

(b) exposing the cells to a substrate of β-lactamase that is a cell-permeable dye that contains two fluorescent moieties where the two fluorescent moieties are on different parts of the dye and cleavage of the dye by β-lactamase allows the two fluorescent moietites to drift apart;

(c) measuring the amount of fluorescent resonance energy transfer (FRET) in the cells in the absence of the substance of step (d);

(d) exposing the cells to a substance that is suspected of being an agonist of the $GABA_B$ receptor;

(e) measuring the amount of FRET in the cells after exposure of the cells to the substance;

wherein if the amount of FRET in the cells measured in step (e) is less that the amount of FRET measured in the cells in step (c), then the substance is an agonist of the $GABA_B$ receptor.

Substeps (1)-(3) of step (a) can be practiced in any order.

The assay described above can be modified to an assay for identifying antagonists of the $GABA_B$ receptor. Such modification would involve the use of β-lactamase under the control of a promoter that is repressed by at least one intracellular signal generated by interaction of an agonist with the $GABA_B$ receptor and would also involve running the assay in the presence of a known agonist. When the cells are exposed to substances suspected of being antagonists of the $GABA_B$ receptor, β-lactamase will be induced, and FRET will decrease, only if the substance tested is able to counteract the effect of the agonist, *i.e.*, only if the substance tested is acutally an antagonist.

Accordingly, the present invention includes a method for identifying antagonists of the $GABA_B$ receptor comprising:

(a) transfecting cells with:

(1) an expression vector that directs the expression of HG20 in the cells;

(2) an expression vector that directs the expression of $GABA_BR1a$ or $GABA_BR1b$ in the cells;

(3) an expression vector that directs the expression of β-lactamase under the control of an inducible promoter that is repressed by at least one intracellular signal generated by interaction of an agonist with the $GABA_B$ receptor;

(b) exposing the cells to a known agonist of the $GABA_B$ receptor;

(c) exposing the cells to a substrate of β-lactamase that is a cell-permeable dye that contains two fluorescent moieties where the two fluorescent moieties are on different parts of the dye and cleavage of the dye by β-lactamase allows the two fluorescent moietites to drift apart;

(d) measuring the amount of fluorescent resonance energy transfer (FRET) in the cells in the absence of the substance of step (e);

(e) exposing the cells to a substance that is suspected of being an antagonist of the $GABA_B$ receptor;

(f) measuring the amount of FRET in the cells after exposure of the cells to the substance;

wherein if the amount of FRET in the cells measured in step (f) is less that the amount of FRET measured in the cells in step (d), then the substance is an antagonist of the $GABA_B$ receptor.

Substeps (1)-(3) of step (a) can be practiced in any order.

In particular embodiments of the assays employing β-lactamase described above, the cells are eukaryotic cells. In particular embodiments, the cells are mammalian cells. In particular embodiments, the cells are selected from the group consisting of: L cells L-M(TK$^-$) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), *Xenopus* melanophores, and *Xenopus* oocytes.

In other embodiments, the inducible promoter that is repressed by at least one intracellular signal generated by interaction of an agonist with the $GABA_B$ receptor is a promoter that is repressed by decreases in cAMP levels or changes in potassium currents.

In other embodiments, the inducible promoter that is activated by at least one intracellular signal generated by interaction of an agonist with the $GABA_B$ receptor is a promoter that is activated by decreases in cAMP levels or changes in potassium currents.

In other emebodiments, the known agonist is selected from the group consisting of: GABA, saclofen, (-)baclofen, glycine, and (L)-glutamic acid.

In other embodiments, β-lactamase is TEM-1 β-lactamase from *Escherichia coli*.

In other embodiments, the subtrate of β-lactamase is CCF2/AM (Zlokarnik et al., 1998, Science 279:84-88).

In other embodiments, HG20 has an amino acid sequence of SEQ.ID.NO.:2.

In other embodiments of the above-described methods, HG20 comprises an amino acid sequence selected from the group consisting of:

SEQ.ID.NO.:2;
Positions 9-941 of SEQ.ID.NO.:2;
Positions 35-941 of SEQ.ID.NO.:2;
Positions 36-941 of SEQ.ID.NO.:2;
Positions 38-941 of SEQ.ID.NO.:2;
Positions 39-941 of SEQ.ID.NO.:2;
Positions 42-941 of SEQ.ID.NO.:2;
Positions 44-941 of SEQ.ID.NO.:2;
Positions 46-941 of SEQ.ID.NO.:2;
Positions 52-941 of SEQ.ID.NO.:2; and
Positions 57-941 of SEQ.ID.NO.:2.

In other embodiments, $GABA_BR1a$ is murine $GABA_BR1a$ and has the amino acid sequence SEQ.ID.NO.:20. In other embodiments, $GABA_BR1a$ is rat $GABA_BR1a$ and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In other embodiments, $GABA_BR1b$ is rat $GABA_BR1b$ and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In other embodiments, $GABA_BR1a$ is human $GABA_BR1a$ and has an amino acid sequence selected from the group consisting of: SEQ.ID.NO.:21 and the protein encoded by SEQ.ID.NO.:23.

In particular embodiments, the cells express a promiscuous G-protein, *e.g.*, Gα15 or Gα16.

In particular embodiments, the inducible promoter is a promoter that is activated or repressed by NF-κB or NFAT.

The assays descibed above could be modified to identify inverse agonists. In such assays, one would expect a decrease in β-lactamase activity. Similarly, inverse agonists can be identified by modifying the functional assays that were described previously where those functional assays monitored decreases in cAMP levels. In the case

of assays for inverse agonists, increases in cAMP levels would be observed.

Other transcription-based assays that can be used to identify agonists and antagonists of the $GABA_B$ receptor rely on the use of green fluorescent proteins or luciferase as reported genes. An example of such an assay comprises:

(a) transfecting cells with:

(1) an expression vector that directs the expression of HG20 in the cells;

(2) an expression vector that directs the expression of $GABA_B$R1a or $GABA_B$R1b in the cells;

(3) an expression vector that directs the expression of green flurorescent protein (GFP) under the control of an inducible promoter that is activated by an intracellular signal generated by the interaction of agonists and the $GABA_B$ receptor;

(b) measuring the amount of fluorescence from GFP in the cells;

(c) exposing the cells to a substance that is suspected of being an agonist of the $GABA_B$ receptor;

(d) measuring the amount of fluorescence from GFP in the cells that have been exposed to the substance;

wherein if the amount of fluorescence from GFP in the cells measured in step (b) is less that the amount of fluorescence from GFP measured in the cells in step (d), then the substance is an agonist of the $GABA_B$ receptor.

The present invention also includes assays for the identification of agonists or antagonists of $GABA_B$ receptors that are based upon FRET between a first and a second fluorescent dye where the first dye is bound to one side of the plasma membrane of a cell expressing a heterodimer of HG20 and $GABA_B$R1a or $GABA_B$R1b and the second dye is free to shuttle from one face of the membrane to the other face in response to changes in membrane potential. In certain embodiments, the first dye is impenetrable to the plasma membrane of the cells and is bound predominately to the extracellular surface of the plasma

membrane. The second dye is trapped within the plasma membrane but is free to diffuse within the membrane. At normal (*i.e.*, negative) resting potentials of the membrane, the second dye is bound predominately to the inner surface of the extracellular face of the plasma membrane, thus placing the second dye in close proximity to the first dye. This close proximity allows for the generation of a large amount of FRET between the two dyes. Following membrane depolarization, the second dye moves from the extracellular face of the membrane to the intracellular face, thus increasing the distance between the dyes. This increased distance results in a decrease in FRET, with a corresponding increase in fluorescent emission derived from the first dye and a corresponding decrease in the fluorescent emission from the second dye. See figure 1 of González & Tsien, 1997, Chemistry & Biology 4:269-277. See also González & Tsien, 1995, Biophys. J. 69:1272-1280 and U.S. Patent No. 5,661,035.

In certain embodiments, the first dye is a fluorescent lectin or a fluorescent phospholipid that acts as the fluorescent donor. Examples of such a first dye are: a coumarin-labeled phosphatidylethanolamine (*e.g.*, N-(6-chloro-7-hydroxy-2-oxo-2H--1-benzopyran-3-carboxamidoacetyl)-dimyristoylphosphatidyl-ethanolamine) or N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-dipalmitoylphosphatidylethanolamine); a fluorescently-labeled lectin (*e.g.*, fluorescein-labeled wheat germ agglutinin). In certain embodiments, the second dye is an oxonol that acts as the fluorescent acceptor. Examples of such a second dye are: bis(1,3-dialkyl-2-thiobarbiturate)trimethineoxonols (*e.g.*, bis(1,3-dihexyl-2-thiobarbiturate)trimethineoxonol) or pentamethineoxonol analogues (*e.g.*, bis(1,3-dihexyl-2-thiobarbiturate)pentamethineoxonol; or bis(1,3-dibutyl-2-thiobarbiturate)pentamethineoxonol). See González & Tsien, 1997, Chemistry & Biology 4:269-277 for methods of synthesizing various dyes suitable for use in the present invention. In certain embodiments, the assay may comprise a natural carotenoid, *e.g.*, astaxanthin, in order to reduce photodynamic damage due to singlet oxygen.

Accordingly, the present invention provides a method of identifying agonists of $GABA_B$ receptors comprising:

(a) providing test cells comprising:

(1) an expression vector that directs the expression of HG20 in the cells;

(2) an expression vector that directs the expression of $GABA_BR1a$ or $GABA_BR1b$ in the cells;

(3) an expression vector that directs the expression of an inwardly rectifying potassium channel;

(4) a first fluorescent dye, where the first dye is bound to one side of the plasma membrane; and

(5) a second fluorescent dye, where the second fluorescent dye is free to shuttle from one face of the plasma membrane to the other face in response to changes in membrane potential;

(b) exposing the test cells to a substance that is suspected of being an agonist of the $GABA_B$ receptor;

(c) measuring the amount of fluorescence resonance energy transfer (FRET) in the test cells that have been exposed to the substance;

(d) comparing the amount of FRET exhibited by the test cells that have been exposed to the substance with the amount of FRET exhibited by control cells;

wherein if the amount of FRET exhibited by the test cells is less than the amount of FRET exhibited by the control cells, the substance is an agonist of the $GABA_B$ receptor;

where the control cells are either (1) cells that are essentially the same as the test cells except that they do not comprise at least one of the items listed at (a) (1)-(5) but have been exposed to the substance; or (2) test cells that have not been exposed to the substance.

The above-described assay can be easily modified to form a method to identify antagonists of the $GABA_B$ receptor. Such a method comprises:

(a) providing test cells comprising:

(1) an expression vector that directs the expression of HG20 in the cells;

(2) an expression vector that directs the expression of $GABA_BR1a$ or $GABA_BR1b$ in the cells;

(3) an expression vector that directs the expression of an inwardly rectifying potassium channel;

(4) a first fluorescent dye, where the first dye is bound to one side of the plasma membrane; and

(5) a second fluorescent dye, where the second fluorescent dye is free to shuttle from one face of the plasma membrane to the other face in response to changes in membrane potential;

(b) exposing the test cells to a known agonist of the $GABA_B$ receptor in the presence of a substance that is suspected of being an antagonist of the $GABA_B$ receptor;

(c) exposing the test cells to the known agonist of the $GABA_B$ receptor in the absence of the substance that is suspected of being an antagonist of the $GABA_B$ receptor;

(d) measuring the amount of fluorescence resonance energy transfer (FRET) in the test cells of steps (b) and (c);

(e) comparing the amount of FRET exhibited by the test cells of steps (b) and (c);

where if the amount of FRET exhibited by the test cells of step (b) is greater than the amount of FRET exhibited by the test cells of step (c), the substance is an antagonist of the $GABA_B$ receptor.

In particular embodiments of the above-described methods, the expression vectors are transfected into the test cells.

In particular embodiments of the above-described methods, HG20 has an amino acid sequence of SEQ.ID.NO.:2.

In particular embodiments of the above-described methods, HG20 comprises an amino acid sequence selected from the group consisting of:

SEQ.ID.NO.:2;

Positions 9-941 of SEQ.ID.NO.:2;

Positions 35-941 of SEQ.ID.NO.:2;

Positions 36-941 of SEQ.ID.NO.:2;

Positions 38-941 of SEQ.ID.NO.:2;

Positions 39-941 of SEQ.ID.NO.:2;

Positions 42-941 of SEQ.ID.NO.:2;

Positions 44-941 of SEQ.ID.NO.:2;
Positions 46-941 of SEQ.ID.NO.:2;
Positions 52-941 of SEQ.ID.NO.:2; and
Positions 57-941 of SEQ.ID.NO.:2.

In particular embodiments of the above-described methods, GABA$_B$R1a is murine GABA$_B$R1a and has the amino acid sequence SEQ.ID.NO.:20. In particular embodiments, GABA$_B$R1a is rat GABA$_B$R1a and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In particular embodiments, GABA$_B$R1b is rat GABA$_B$R1b and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In particular embodiments, GABA$_B$R1a is human GABA$_B$R1a and has an amino acid sequence selected from the group consisting of: SEQ.ID.NO.:21 and the protein encoded by SEQ.ID.NO.:23.

Inwardly rectifying potassium channels that are suitable for use in the methods of the present invention are disclosed in, *e.g.*, Misgeld et al., 1995, Prog. Neurobiol. 46:423-462; North, 1989, Br. J. Pharmacol. 98:13-23; Gahwiler et al.,1985, Proc. Natl. Acad. Sci USA 82:1558-1562; Andrade et al., 1986, Science 234:1261.

In particular embodiments of the above-described methods, the first fluorescent dye is selected from the group consisting of: a fluorescent lectin; a fluorescent phospholipid; a coumarin-labeled phosphatidylethanolamine; N-(6-chloro-7-hydroxy-2-oxo-2H--1-benzopyran-3-carboxamidoacetyl)-dimyristoylphosphatidyl-ethanolamine); N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-dipalmitoylphosphatidylethanolamine); and fluorescein-labeled wheat germ agglutinin.

In particular embodiments of the above-described methods, the second fluorescent dye is selected from the group consisting of: an oxonol that acts as the fluorescent acceptor; bis(1,3-dialkyl-2-thiobarbiturate)trimethineoxonols; bis(1,3-dihexyl-2-thiobarbiturate)trimethineoxonol; bis(1,3-dialkyl-2-thiobarbiturate)quatramethineoxonols; bis(1,3-dialkyl-2-thiobarbiturate)pentamethineoxonols; bis(1,3-dihexyl-2-

thiobarbiturate)pentamethineoxonol; bis(1,3-dibutyl-2-thiobarbiturate)pentamethineoxonol); and bis(1,3-dialkyl-2-thiobarbiturate)hexamethineoxonols.

In a particular embodiment of the above-described methods, the cells are eukaryotic cells. In another embodiment, the cells are mammalian cells. In other embodiments, the cells are L cells L-M(TK$^-$) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), *Xenopus* melanophores, or *Xenopus* oocytes.

In a particular embodiment of the above-described methods, the cells are transfected with separate expression vectors that direct the expression of HG20 and either $GABA_BR1a$ or $GABA_BR1b$ in the cells. In other embodiments, the cells are transfected with a single expression vector that direct the expression of both HG20 and $GABA_BR1a$ or $GABA_BR1b$ in the cells.

The conditions under which step (b) of the first method described above and steps (b) and (c) of the second method described above are practiced are conditions that are typically used in the art for the study of protein-ligand interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

The $GABA_B$ receptor belongs to the class of proteins known as G-protein coupled receptors (GPCRs). GPCRs transmit signals across cell membranes upon the binding of ligand. The ligand-bound GPCR interacts with a heterotrimeric G-protein, causing the $G\alpha$ subunit of the G-protein to disassociate from the $G\beta$ and $G\gamma$ subunits. The $G\alpha$ subunit can then go on to activate a variety of second messenger systems.

Generally, a particular GPCR is only coupled to a particular type of G-protein. Thus, to observe a functional response from the GPCR, it is necessary to ensure that the proper G-protein is present in the system containing the GPCR. It has been found, however, that there are certain

G-proteins that are "promiscuous." These promiscuous G-proteins will couple to, and thus transduce a functional signal from, virtually any GPCR. See Offermanns & Simon, 1995, J. Biol. Chem. 270:15175, 15180 (Offermanns). Offermanns described a system in which cells are transfected with expression vectors that result in the expression of one of a large number of GPCRs as well as the expression of one of the promiscuous G-proteins Gα15 or Gα16. Upon the addition of an agonist of the GPCR to the transfected cells, the GPCR was activated and was able, via Gα15 or Gα16, to activate the β isoform of phospholipase C, leading to an increase in inositol phosphate levels in the cells.

Therefore, by making use of these promiscuous G-proteins as in Offermanns, it is possible to set up functional assays for the $GABA_B$ receptor, even in the absence of knowledge of the G-protein with which the $GABA_B$ receptoris coupled *in vivo*. One possibility for utilizing promiscuous G-proteins in connection with the $GABA_B$ receptor includes a method of identifying agonists of the $GABA_B$ receptorcomprising:

(a) providing cells that express HG20, $GABA_BR1a$ or $GABA_BR1b$, and a promiscuous G-protein, where HG20 and either $GABA_BR1a$ or $GABA_BR1b$ form a heterodimer representing a functional $GABA_B$ receptor;

(b) exposing the cells to a substance that is a suspected agonist of the $GABA_B$ receptor;

(c) measuring the level of inositol phosphates in the cells;

where an increase in the level of inositol phosphates in the cells as compared to the level of inositol phosphates in the cells in the absence of the suspected agonist indicates that the substance is an agonist of the $GABA_B$ receptor.

Levels of inositol phosphates can be measured by monitoring calcium mobilization. Intracellular calcium mobilization is typically assayed in whole cells under a microscope using fluorescent dyes or in cell suspensioins via luminescence using the aequorin assay.

In methods related to those described above, rather than using changes in inositol phosphate levels as an indication of $GABA_B$ receptorfunction, potassium currents are measured. This is feasible since

the $GABA_B$ receptor, like other metabotropic receptors, is expected to be coupled to potassium channels. Thus, one could measure $GABA_B$ receptor coupling to GIRK2 channels or to other potassium channels in oocytes.

In a particular embodiment of the above-described method, the cells are eukaryotic cells. In another embodiment, the cells are mammalian cells. In other embodiments, the cells are L cells L-M(TK$^-$) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), or Xenopus oocytes.

In a particular embodiment of the above-described method, the cells are transfected with expression vectors that direct the expression of HG20, $GABA_B$R1a or $GABA_B$R1b, and the promiscuous G-protein in the cells.

The conditions under which step (b) of the method is practiced are conditions that are typically used in the art for the study of protein-ligand interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

In a particular embodiment of the above-described method, the promiscuous G-protein is selected from the group consisting of $G\alpha 15$ or $G\alpha 16$. Expression vectors containing $G\alpha 15$ or $G\alpha 16$ are known in the art. See, *e.g.*, Offermanns; Buhl et al., 1993, FEBS Lett. 323:132-134; Amatruda et al., 1993, J. Biol. Chem. 268:10139-10144.

The above-described assay can be easily modified to form a method to identify antagonists of the $GABA_B$ receptor. Such a method is also part of the present invention and comprises:

(a) providing cells that express HG20, $GABA_B$R1a or $GABA_B$R1b, and a promiscuous G-protein;

(b) exposing the cells to a substance that is an agonist of the $GABA_B$ receptor;

(c) subsequently or concurrently to step (b), exposing the cells to a substance that is a suspected antagonist of the $GABA_B$ receptor;

(d) measuring the level of inositol phosphates in the cells;

where a decrease in the level of inositol phosphates in the cells in the presence of the suspected antagonist as compared to the level of inositol phosphates in the cells in the absence of the suspected antagonist indicates that the substance is an antagonist of the $GABA_B$ receptor.

In a particular embodiment of the above-described method, the agonist is an amino acid such as GABA, glutamate, glycine, or amino acid analogues such as (-)baclofen.

In a particular embodiment of the above-described method, the cells are eukaryotic cells. In another embodiment, the cells are mammalian cells. In other embodiments, the cells are L cells L-M(TK^-) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), HEK293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), or Xenopus oocytes.

The conditions under which steps (b) and (c) of the method are practiced are conditions that are typically used in the art for the study of protein-ligand interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

In a particular embodiment of the above-described method, the cells are transfected with expression vectors that direct the expression of HG20, $GABA_B$R1a or $GABA_B$R1b, and the promiscuous G-protein in the cells.

In a particular embodiment of the above-described method, the promiscuous G-protein is selected from the group consisting of $G\alpha15$ or $G\alpha16$.

In particular embodiments of the above-described methods, HG20 has an amino acid sequence of SEQ.ID.NO.:2.

In other embodiments of the above-described methods, HG20 comprises an amino acid sequence selected from the group consisting of:

SEQ.ID.NO.:2;

Positions 9-941 of SEQ.ID.NO.:2;

Positions 35-941 of SEQ.ID.NO.:2;

Positions 36-941 of SEQ.ID.NO.:2;

Positions 38-941 of SEQ.ID.NO.:2;

Positions 39-941 of SEQ.ID.NO.:2;

Positions 42-941 of SEQ.ID.NO.:2;

Positions 44-941 of SEQ.ID.NO.:2;

Positions 46-941 of SEQ.ID.NO.:2;

Positions 52-941 of SEQ.ID.NO.:2; and

Positions 57-941 of SEQ.ID.NO.:2.

In other embodiments, $GABA_BR1a$ is murine $GABA_BR1a$ and has the amino acid sequence SEQ.ID.NO.:20. In other embodiments, $GABA_BR1a$ is rat $GABA_BR1a$ and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In other embodiments, $GABA_BR1b$ is rat $GABA_BR1b$ and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In other embodiments, $GABA_BR1a$ is human $GABA_BR1a$ and has an amino acid sequence selected from the group consisting of: SEQ.ID.NO.:21 and the protein encoded by SEQ.ID.NO.:23.

While the above-described methods are explicitly directed to testing whether "a" substance is an agonist or antagonist of the $GABA_B$ receptor, it will be clear to one skilled in the art that such methods can be adapted to test collections of substances, *e.g.*, combinatorial libraries, to determine whether any members of such collections are activators or inhibitors of the $GABA_B$ receptor. Accordingly, the use of collections of substances, or individual members of such collections, as the substance in the above-described methods is within the scope of the present invention.

The present invention includes pharmaceutical compositions comprising agonists and antagonists of $GABA_B$ receptors that have been identified by the above-described methods. The agonists and antagonists are generally combined with pharmaceutically acceptable carriers to form

pharmaceutical compositions. Examples of such carriers and methods of formulation of pharmaceutical compositions containing agonists and antagonists and carriers can be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain a therapeutically effective amount of the agonists and antagonists.

Therapeutic or prophylactic compositions are administered to an individual in amounts sufficient to treat or prevent conditions where $GABA_B$ receptor activity is abnormal. The effective amount can vary according to a variety of factors such as the individual's condition, weight, gender, and age. Other factors include the mode of administration. The appropriate amount can be determined by a skilled physician.

Compositions can be used alone at appropriate dosages. Alternatively, co-administration or sequential administration of other agents can be desirable.

The compositions can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compositions can be administered in such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection. Likewise, they can also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts.

Advantageously, compositions can be administered in a single daily dose, or the total daily dosage can be administered in divided doses of two, three or four times daily. Furthermore, compositions can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

The dosage regimen utilizing the compositions is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal, hepatic and cardiovascular function of the patient; and the particular composition thereof employed. A physician of ordinary skill can readily determine and prescribe the effective amount of the composition required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentrations of composition within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the composition's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of a composition.

Agonists and antagonists identified by the above-described methods are useful in the same manner as well-known agonists and antagonists of other $GABA_B$ receptors. For example, (-) baclofen is a known agonist of $GABA_B$ receptors and, in racemic form, is a clinically useful muscle relaxant known as LIORESAL® (Bowery & Pratt, 1992, Arzneim.-Forsch./Drug Res. 42:215-223 [Bowery & Pratt]). Similarly, the agonists and antagonists of $GABA_B$ receptors identified by the methods of the present invention are expected to be useful as muscle relaxants. Bowery & Pratt, at Table 1, page 219, list the therapeutic potential of $GABA_B$ receptor agonists and antagonists. For agonists, the therapeutic potential is said to include use as muscle relaxants and anti-asthmatics. For antagonists, the therapeutic potential is said to include use as antidepressants, anticonvulsants, nootropics, and anxiolytics. Additionally, at page 220, left column, Bowery & Pratt list some additional therapeutic uses for the $GABA_B$ receptor agonist (-) baclofen: treatment of trigeminal neuralgia and reversal of ethanol withdrawal symptoms. Given the wide range of utility displayed by known agonists and antagonists of $GABA_B$ receptors, it is clear that those skilled in the art would consider the agonists and antagonists identified by the methods of the present invention to be pharamacologically useful. In addition, it is believed that such agonists and antagonists will also be useful in the treatment of epilepsy, neuropsychiatric disorders, and dementias.

When screening compounds in order to identify potential pharmaceuticals that specifically interact with a target receptor, it is necessary to ensure that the compounds identified are as specific as possible for the target receptor. To do this, it is necessary to screen the compounds against as wide an array as possible of receptors that are similar to the target receptor. Thus, in order to find compounds that are potential pharmaceuticals that interact with receptor A, it is necessary not only to ensure that the compounds interact with receptor A (the "plus target") and produce the desired pharmacological effect through receptor A, it is also necessary to determine that the compounds do not interact with receptors B, C, D, etc (the "minus targets"). In general, as part of a screening program, it is important to have as many minus targets as possible (see Hodgson, 1992, Bio/Technology 10:973-980, at 980). HG20 protein, DNA encoding HG20 protein, $GABA_BR1a$ protein, DNA encoding $GABA_BR1a$ protein,and recombinant cells that have been engineered to express HG20 protein and $GABA_BR1a$ protein have utility in that they can be used as "minus targets" in screens design to identify compounds that specifically interact with other G-protein coupled receptors, *i.e.*, non-$GABA_B$ receptors.

The present invention also includes antibodies to the HG20 protein. Such antibodies may be polyclonal antibodies or monoclonal antibodies. The antibodies of the present invention are raised against the entire HG20 protein or against suitable antigenic fragments of the protein that are coupled to suitable carriers, *e.g.*, serum albumin or keyhole limpet hemocyanin, by methods well known in the art. Methods of identifying suitable antigenic fragments of a protein are known in the art. See, *e.g.*, Hopp & Woods, 1981, Proc. Natl. Acad. Sci. USA 78:3824-3828; and Jameson & Wolf, 1988, CABIOS (Computer Applications in the Biosciences) 4:181-186. Particularly suitable peptides are: amino acids 357-371 of SEQ.ID.NO.:2 and amino acids 495-511 of SEQ.ID.NO.:2. Also, anti-peptide antisera can be generated by immunization of New Zealand White rabbits with a KLH-conjugation of a 20 amino acid synthetic peptide corresponding to residues 283-302 of HG20 (GWYEPSWWEQVHTEANSSRC) (a portion of SEQ.ID.NO.:2).

For the production of polyclonal antibodies, HG20 protein or an antigenic fragment, coupled to a suitable carrier, is injected on a periodic basis into an appropriate non-human host animal such as, *e.g.*, rabbits, sheep, goats, rats, mice. The animals are bled periodically and sera obtained are tested for the presence of antibodies to the injected antigen. The injections can be intramuscular, intraperitoneal, subcutaneous, and the like, and can be accompanied with adjuvant.

For the production of monoclonal antibodies, HG20 protein or an antigenic fragment, coupled to a suitable carrier, is injected into an appropriate non-human host animal as above for the production of polyclonal antibodies. In the case of monoclonal antibodies, the animal is generally a mouse. The animal's spleen cells are then immortalized, often by fusion with a myeloma cell, as described in Kohler & Milstein, 1975, Nature 256:495-497. For a fuller description of the production of monoclonal antibodies, see <u>Antibodies: A Laboratory Manual</u>, Harlow & Lane, eds., Cold Spring Harbor Laboratory Press, 1988.

Gene therapy may be used to introduce HG20 polypeptides into the cells of target organs. Nucleotides encoding HG20 polypeptides can be ligated into viral vectors which mediate transfer of the nucleotides by infection of recipient cells. Suitable viral vectors include retrovirus, adenovirus, adeno-associated virus, herpes virus, vaccinia virus, and polio virus based vectors. Alternatively, nucleotides encoding HG20 polypeptides can be transferred into cells for gene therapy by non-viral techniques including receptor-mediated targeted transfer using ligand-nucleotide conjugates, lipofection, membrane fusion, or direct microinjection. These procedures and variations thereof are suitable for *ex vivo* as well as *in vivo* gene therapy. Gene therapy with HG20 polypeptides will be particularly useful for the treatment of diseases where it is beneficial to elevate HG20 activity.

The following non-limiting examples are presented to better illustrate the invention.

EXAMPLE 1

Cloning and sequencing of HG20

A cDNA fragment encoding full-length HG20 can be isolated from a human fetal brain cDNA library by using the polymerase chain reaction (PCR) employing the following primer pair:

HG20.F139 5'-CCGTTCTGAGCCGAGCCG -3' (SEQ.ID.NO.:3)
HG20.R3195 5'-TCCGCAGCCAGAGCCGACAG-3' (SEQ.ID.NO.:4)

The above primer pair is meant to be illustrative only. Those skilled in the art would recognize that a large number of primer pairs, based upon SEQ.ID.NO.:1, could also be used.

PCR reactions can be carried out with a variety of thermostable enzymes including but not limited to AmpliTaq, AmpliTaq Gold, Vent polymerase. For AmpliTaq, reactions can be carried out in 10 mM Tris-Cl, pH 8.3, 2.0 mM $MgCl_2$, 200 μM for each dNTP, 50 mM KCl, 0.2 μM for each primer, 10 ng of DNA template, 0.05 units/μl of AmpliTaq. The reactions are heated at 95°C for 3 minutes and then cycled 35 times using the cycling parameters of 95°C, 20 seconds, 62°C, 20 seconds, 72°C, 3 minutes. In addition to these conditions, a variety of suitable PCR protocols can be found in PCR Primer, A Laboratory Manual, edited by C.W. Dieffenbach and G.S. Dveksler, 1995, Cold Spring Harbor Laboratory Press.

A suitable cDNA library from which a clone encoding HG20 can be isolated would be a random primed fetal brain cDNA library consisting of approximately 4.0 million primary clones constructed in the plasmid vector pBluescript (Stratagene, LaJolla, CA). The primary clones of such a library can be subdivided into pools with each pool containing approximately 20,000 clones and each pool can be amplified separately.

By this method, a cDNA fragment (SEQ.ID.NO.:1) encoding an open reading frame of 941 amino acids (SEQ.ID.NO.:2) is obtained. This cDNA fragment can be cloned into a suitable cloning vector or

expression vector. For example, the fragment can be cloned into the mammalian expression vector pcDNA3.1 (Invitrogen, San Diego, CA). HG20 protein can then be produced by transferring an expression vector containing SEQ.ID.NO.:1 or portions thereof into a suitable host cell and growing the host cell under appropriate conditions. HG20 protein can then be isolated by methods well known in the art.

Alternatively, other cDNA libraries made from human tissues that express HG20 RNA can be used with PCR primers HG20.F139 and HG20.R3195 in order to amplify a cDNA fragment encoding full-length HG20. Suitable cDNA libraries would be those prepared from cortex, cerebellum, testis, ovary, adrenal gland, thyroid, or spinal cord.

As an alternative to the above-described PCR method, a cDNA clone encoding HG20 can be isolated from a cDNA library using as a probe oligonucleotides specific for HG20 and methods well known in the art for screening cDNA libraries with oligonucleotide probes. Such methods are described in, *e.g.*, Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, *DNA Cloning: A Practical Approach*, MRL Press, Ltd., Oxford, U.K., Vol. I, II. Oligonucleotides that are specific for HG20 and that can be used to screen cDNA libraries are:

HG20.F46	5'-GGGATGATCATGGCCAGTGC-3' (SEQ.ID.NO.:5)
HG20.R179	5'-GGATCCATCAAGGCCAAAGA-3' (SEQ.ID.NO.:6)
HG21.F43	5'-GCCGCTGTCTCCTTCCTGA-3' (SEQ.ID.NO.:7)
HG21.R251	5'-TTGGTTCACACTGGTGACCGA-3' (SEQ.ID.NO.:8)
HG20.R123	5'-TTCACCTCCCTGCTGTCTTG-3' (SEQ.ID.NO.:9)
HG20.F1100	5'-CAGGCGATTCCAGTTCACTCA-5' (SEQ.ID.NO.:10)
HG20.F1747	5'-GAACCAAGCCAGCACATCCC-3' (SEQ.ID.NO.:11)
HG20.R54	5'-CCTCGCCATACAGAACTCC-3' (SEQ.ID.NO.:12)
HG20.R75	5'-GTGTCATAGAGCCGCAGGTC-3' (SEQ.ID.NO.:13)
HG20.F139	5'-CCGTTCTGAGCCGAGCCG-3' (SEQ.ID.NO.:3)
HG20.R3195	5'-TCCGCAGCCAGAGCCGACAG-3' (SEQ.ID.NO.:4)

Membrane-spanning proteins, such as $GABA_B$ receptors, when first translated generally possess an approximately 16 to 40 amino acid segment known as a signal sequence. Signal sequences direct the nascent protein to be transported through the endoplasmic reticulum membrane, following which signal sequences are cleaved from the protein. Signal sequences generally contain from 4 to 12 hydrophobic residues but otherwise possess little sequence homology. The Protein Analysis tool of the GCG program (Genetics Computer Group, Madison, Wisconsin), a computer program capable of identifying likely signal sequences, was used to examine the N terminus of HG20. Several likely candidates for cleavage sites which would generate mature HG20 protein, *i.e.*, protein lacking the signal sequence, were identified. The results are shown in Figure 3.

EXAMPLE 2

Expression of HG20 in normal and diseased adrenal tissue

Northern blots were performed to measure the amount of HG20 RNA in normal and diseased adrenal tissue. The results are shown in Table 2 below. The amount of the approximately 6.5 kb HG20 transcript is shown normalized to the amount of β-actin transcript.

Table 2

Pathology	Profile	HG20 RNA	Actin RNA	HG20 /actin
Pheochromocytoma	M, 30 yr	0.47	0.74	0.64
Adrenal carcinoma cortex	M, 69 yr	0.61	0.80	0.76
Adrenal adenoma cortex	M, 69 yr	0.62	1.15	0.54
Normal Adrenal	M, 26 yr	1.00	1.00	1.00

The results shown in Table 2 indicate that HG20 expression is decreased in diseased states of the adrenal gland. Thus, increasing the

concentration of HG20 in such diseased states is likely to be pharmacologically useful. Accordingly, one skilled in the art would expect agonists of HG20 to be pharmacologically useful.

EXAMPLE 3

Tissue distribution of various HG20 RNA transcripts

Table 3, below, shows the results of experiments to measure the amount of HG20 RNA transcripts of various lengths in various tissues. The results shown were derived from a multiple tissue Northern blot that was hybridized overnight in expressHyb solution (Clontech). Washing conditions were: 0.1X SSC, 0.1% SDS, at 60°C. A ^{32}P-random primer labelled Eco RI fragment containing the full-length native HG20 DNA was used as a hybridization probe. The greater the number of plus signs in a particular tissue, the greater was the amount of HG20 RNA detected in that tissue.

Table 3

Tissue	6.5 kb	4.5 kb	4.0 kb	1.8 kb
cerebellum	++	+		
cerebral cortex	++++	+		
medulla	+	+		
occipital pole	+	+		
frontal lobe	+++	+		
temporal lobe	+++	+		
putamen	++	+		
spinal cord n=2	++	+		
amygdala	+++			
caudate nucleus	+	+		
corpus callosum	+	+		
hippocampus	++	+		
whole brain	+++	+		
substantia nigra	+	+		
subthalamic nucleus	+	+		
thalamus	++	+		

spleen		+		
thymus n=2		++		
prostate		++		
testis n=2	++	+	+++	
ovary		++	+	+
small intestine n=2		++		
colon (mucosal lining)		++		
peripheral blood leucocytes		++		
stomach n=2	+	+		
thyroid n=2	++	++++		
lymph node		+		
trachea		++		
adrenal gland	+++	+++	+	++++
bone marrow		++		
heart	+	++		
brain	+++++			
placenta		+		
lung		+		
liver		+		
skeletal muscle	+	++		
kidney		+		
pancreas	+	+		
adrenal medulla	+++			+
adrenal cortex	+++++		++	++

The distribution of HG20 RNA shown in Table 3 suggests that HG20 mediates activities of the central and peripheral nervous system.

EXAMPLE 4

<u>Distribution of HG20 mRNA in brain</u>

Using in situ hybridisation, the distribution of HG20 mRNA in squirrel monkey brain was studied. Antisense oligonucleotide probes to HG20 were generated on an Applied Biosystems Model 394 DNA synthesiser and purified by preparative polyacrylamide electrophoresis.

Probe 1: 5'ATC-TGG-GTT-TGT-TCT-CAG-GGT-GAT-GAG-CTT-CGG-CAC-GAA-TAC-CAG 3' (SEQ.ID.NO.:14);
Probe2: 5' GCT-CTG-TGA-TCT-TCA-TTC-GCA-GGC-GAT-GGT-TTT-CTG-ACT-GTA-GGC 3' (SEQ.ID.NO.:15).

Each oligonucleotide was 3'-end labelled with [^{35}S] deoxyadenosine 5'-(thiotriphosphate) in a 30:1 molar ratio of ^{35}S-isotope:oligonucleotide using terminal deoxynucleotidyl transferase for 15 min at 37°C in the reaction buffer supplied (Boehringer). Radiolabelled oligonucleotide was separated from unincorporated nucleotides using Sephadex G50 spin columns. The specific activities of the labelled probes in several labelling reactions varied from 1.2-2.3 x 10^9 cpm/mg. Squirrel monkey brains were removed and fresh frozen in 1 cm blocks. 12 mm sections were taken and fixed for *in situ* hybridisation. Hybridisation of the sections was carried out according to the method of Sirinathsinghji et al., 1993, Neuroreports 4:175-178. Briefly, sections were removed from alcohol, air dried and 5 x10^5 cpm of each ^{35}S-labelled probe (both oligonucleotides) in 100 ml of hybridisation buffer was applied to each slide. Labelled "antisense" probe was also used in the presence of an excess (100x) concentration of unlabelled antisense probe to define non-specific hybridisation. Parafilm coverslips were placed over the sections which were incubated overnight (about 16 hr) at 37°C. Following hybridisation the sections were washed for 1 hr at 57°C in 1xSSC, then rinsed briefly in 0.1xSSC, dehydrated in a series of alcohols, air dried, and exposed to Amersham Hyperfilm bmax X-ray film. Autoradiographs were analysed using a MCID computerised image analysis system (Image Research Inc., Ontario, Canada).

Highest levels of mRNA for HG20 were found in the hippocampus (dentate gyrus, CA3, CA2, and CA1). High levels were also seen in cortical regions (frontal, cingulate, temporal parietal, entorhinal, and visual) and the cerebellum, although medial septum, thalamic nuclei (medial-dorsal and lateral posterior), lateral geniculates, red nucleus, reticular formation, and griseum pontis all show expression of message. While there are many similiarities with the distribution reported for the $GABA_B$ receptor mRNA in rat, one marked difference is that expression of HG20 mRNA in the monkey caudate and putamen is below the level of

detection while cortical levels are high. In the rat, the GABA$_B$ receptor mRNA appears equally expressed in striatum as in cortex. Figure 4 illustrates these results.

EXAMPLE 5

Attempted recombinant expression of full-length HG20 protein

Following the cloning of HG20 DNA, attempts were made to express full-length HG20 protein (941 amino acids) using various eukaryotic cell lines and expression vectors. The cell lines that were used were: COS-7 cells, HEK293 cells, and frog melanophores. The expression vectors that were used to attempt to express the full-length HG20 protein were: pCR3.1 and pcDNA3.1 (Invitrogen, San Diego, CA) and pciNEO (promega)

All of the attempts to express full-length HG20 described above were unsuccessful. See, *e.g.*, Figure 7, second bar from the left, marked "HG20." See also Figure 5A, lane 1. Although the reason for these failures is not known, it is possible that the highly GC rich nature of the region of the HG20 mRNA that encodes amino acids 1-51 results in the formation of secondary structure in the mRNA that impedes translation. It was only after the construction of an expression vector that encodes a truncated HG20 protein, lacking the first 51 amino acids, that HG20 was successfully expressed. Figure 5A-B shows the results of the successful expression of an HG20 protein having amino acids 52-941. It is expected that expression of HG20 proteins having amino acids 53-941, 54-941, 55-941, *etc.*, could be accomplished in a similar manner. It is also expected that expression of HG20 proteins having the above-described amino termini but having different carboxyl termini could be accomplished in a similar manner as well. Thus, the expression of an HG20 protein having an amino terminus as listed above and having a truncated carboxyl terminus could be accomplished. Alternatively, the carboxyl terminus could be fused to non-HG20 amino acid sequences, forming a chimeric HG20 protein. It is also possible to express HG20

having an amino terminus listed above as a chimeric protein with non-HG20 sequences fused to the amino terminus.

Figure 5A shows the expression of amino acids 52-941 of HG20 as part of a chimeric or fusion protein with the FLAG epitope fused to the amino terminus of the HG20 sequences in a coupled *in vitro* transcription/translation experiment. Figure 5B shows the expression of amino acids 52-941 of HG20 as part of a chimeric or fusion protein with the FLAG epitope fused to the amino terminus of the HG20 sequences in COS-7 cells and melanophores. The expression vector used in this experiment was pcDNA3.1. The expression constructs used in Figure 5A-B also encoded a cleavable signal sequence from the influenza hemaglutinin gene that has been shown to facilitate the membrane insertion of G-protein coupled receptors (Guan et al., 1992, J. Biol. Chem. 267:21995-21998) and the fusion proteins were detected with anti-FLAG antibody. The expression constructs had also been engineered to contain a Kozak consensus sequence prior to the initiating ATG. The amino acid sequences of the hemaglutinin signal sequence and the FLAG epitope were:

[MKTIIALSYIFCLVFA] [DYKDDDDK] SEQ.ID.NO:17
HA signal peptide FLAG epitope

Amino acids 57-941 have been expressed in mammalian cells as part of a chimeric protein. A chimeric construct of HG20 was made that consisted of bases -224 to 99 of the bovine $GABA_A$ $\alpha 1$ gene, a sequence encoding the c-myc epitope tag (amino acid residues 410-419 of the human oncogene product c-myc), a cloning site encoding the amino acid asparagine, and DNA encoding residues 57-941 of HG20. The resultant chimeric protein has the amino acid sequence shown below, with the construct cloned into pcDNA1.1Amp (Invitrogen, San Diego, CA).

____Bovine alpha 1 signal seq______________ __c-myc___ _
MKKSPGLSDYLWAWTLFLSTLTGRSYGQPSLQD EQKLISEEDL N
res. 57-941 HG20

SIMGLMPLT... (SEQ.ID.NO.:18)

The three periods "..." indicate that the chimeric protein sequence extends until amino acid 941 of HG20.

The cell surface expression of this construct was verified using a cell surface ELISA technique. Briefly, HEK293 cells were seeded at ~1x10^5 cells per well in a 24 well tissue culture plate and allowed to adhere for 24 hours. Each well was transfected with a total of 1 µg of DNA. In addition to tagged and un-tagged HG20 constructs, c-myc tagged $GABA_A$ α1 was transfected with $GABA_A$ β1 as a positive control for cell surface expression. Two days after transfection, the cells were assayed for surface expression of the c-myc epitope using the 9E10 monoclonal antibody raised to the c-myc epitope, followed by HRP (horse radish peroxidase) conjugated anti-mouse antibody (Promega) and colormetric development using K-Blue (Bionostics). The results are shown in Figure 7. Figure 7 demoinstrates that when HG20 is part of a chimeric protein, it can be expressed well in mammalian cells but that when attempts are made to express full-length HG20 (amino acids 1-941) directly, *i.e.*, not as part of a chimeric protein, essentially no expression is observed.

EXAMPLE 6

<u>Construction of Full Length Murine $GABA_B$R1a Coding Region</u>

Using a combination of TFASTX (Pearson et al., 1997, Genomics 46:24-36) and TBLASTX (Altschul et al., 1997, Nucleic Acids Res. 25:3389-3402) searching programs against dbEST: Database of Expressed Sequence Tags (URL http://www.ncbi.nlm.nih.gov/dbEST/index.html), we identified partial cDNA clones in the EST collection which encoded murine $GABA_B$R1a using the rat $GABA_B$ receptor subunit cDNAs (GenBank Accession Numbers Y10369 and Y10370) as probe sequences (Kaupmann et al., 1997, Nature 386:239-246). Two of these ESTs (IMAGE Consortium clone identification numbers 472408 and 319196) were obtained (Research Genetics, Birmingham, Ala). The DNA

sequences of both cDNA clones were determined using standard methods on an ABI 373a automated sequencer (Perkin-Elmer-Applied Biosystems, Foster City, CA).

The partial cDNAs were assembled by long accurate PCR using the following oligonucleotides: 472408 sense: 5' - GC GAATTC GGTACC ATG CTG CTG CTG CTG CTG GTG CCT - 3' (SEQ.ID.NO.:24), 472408 antisense: 5' – GG GAATTC TGG ATA TAA CGA GCG TGG GAG TTG TAG ATG TTA AA - 3' (SEQ.ID.NO.:25), 319196 sense: 5' - CCA GAATTC CCA GCC CAA CCT GAA CAA TC - 3' (SEQ.ID.NO.:26), 319196 antisense: 5' - CG GCGGCCGC TCA CTT GTA AAG CAA ATG TA - 3' (SEQ.ID.NO.:27) which amplified two fragments corresponding to the 5' 2,100 basepairs and 3' 1,000 basepairs of the murine $GABA_BR1a$ coding region. The PCR conditions were 200 ng of cDNA template, 2.5 units of Takara LA Taq (PanVera, Madison, WI), 25 mM TAPS (pH 9.3), 50 mM KCl, 2.5 nM $MgCl_2$, 1 mM 2-mercaptoethanol, 100 mM each dNTP and 1 mM each primer with cycling as follows 94°C 1 min, 9 cycles of 98°C for 20 seconds, 72°C-56°C (decreases 2°C per cycle), 72°C for 30 seconds, followed by 30 cycles of 98°C for 20 seconds, 60°C for 3 minutes. A final extension at 72°C for 10 minutes was performed. PCR products were cloned into the TA-Cloning vector pCRII-TOPO (Invitrogen, San Diego, CA) following the manufacturers directions. Cloned PCR products were confirmed by DNA sequencing. To form full-length cDNA, the pCINeo mammalian expression vector was digested with EcoRI and NotI. The EcoRI fragment from PCR cloning of 472408 and the EcoRI/NotI product from PCR cloning of 319196 were ligated in a three part ligation with digested pCINeo vector. The resulting clones were screened by restriction digestion with SstI which cuts once in the vector and once in the 472408 derived fragment. The resulting expression clone is 2,903 basepairs in length. The overall cDNA length, including untranslated sequences, inferred from the full length of the two ESTs is 4,460 basepairs.

EXAMPLE 7

Preparation of membrane fractions

P2 membrane fractions were prepared at 4°C as follows. Tissues or cells were washed twice with cold PBS, collected by centrifugation at 100xg for 7 min, and resuspended in 10 ml of buffer A: 5 mM Tris-HCl, 2 mM EDTA containing (1X) protease inhibitor cocktail Complete® tablets (Boehringer Mannheim), pH 7.4 at 4°C. Tissues or cells were disrupted by polytron homogenization, centrifuged at 100xg for 7 min to pellet unbroken cells and nuclei, and the supernatant collected. The resulting pellet was homogenized a second time in 10 ml of buffer A, centrifuged as described above and supernatant fractions saved. The pooled S1 supernatant was centrifuged at high speed (27 000xg for 20 min) and the pellet was washed once with buffer A, centrifuged (27 000xg for 20 min) and resuspended in buffer A to make the P2 membrane fraction, and stored at -80°C. Protein content was determined using the Bio-Rad Protein Assay Kit according to manufacturer instructions.

EXAMPLE 8

Receptor filter-binding assays

Competition of [^{125}I]CGP71872 binding experiments were performed with ~7 μg P2 membrane protein and increasing concentrations of cold ligand (10^{-12}-10^{-3} M). The concentration of radioligand used in the competition assays was 1 nM (final). Each concentration was examined in duplicate and incubated for 2 hours at 22°C in the dark in a total volume of 250 μL binding buffer: 50 mM Tris-HCl, 2.5 mM $CaCl_2$ (pH 7.4) with (1X) protease inhibitor cocktail Complete® tablets. Bound ligand was isolated by rapid filtration through a Brandel 96 well cell harvester using Whatman GF/B filters. Data were analysed by nonlinear least-squares regression using the computer-fitting program GraphPad Prism version 2.01 (San Diego).

EXAMPLE 9

Photoaffinity labelling

P2 membranes were resuspended in binding buffer and incubated in the dark with 1 nM final concentration [^{125}I]CGP71872 (2200 Ci/mmol) in a final volume of 1 ml for 2 h at 22°C. The membranes were centrifuged at 27,000xg for 10 min and the pellet was washed in ice-cold binding buffer, centrifuged at 27,000xg for 20 min, resuspended in 1 ml of ice-cold binding buffer, and exposed on ice 2 inches from 360 nm ultraviolet light for 10 min. Photolabelled membranes were washed, pelleted by centrifugation, and solubilized in sample buffer (50 mM Tris-HCl pH 6.5, 10% SDS, 10% glycerol, and 0.003% bromophenol blue with 10% 2-mercaptoethanol). Samples were electrophoresed on precast NOVEX 10% Tris-glycine gels, fixed, dried, and exposed to Kodak XAR film with an intensifying screen at -70°C.

EXAMPLE 10

Immunoprecipitation and immunoblotting of $GABA_B$ receptors

Digitonin solubilized FLAG-tagged HG20 receptors were immunoprecipitated with a mouse anti-FLAG M2 antibody affinity resin (Kodak IBI) and immunoblot analysis conducted as previously described (Ng et al., 1996, Biochem. Biophys. Res. Comm. 227:200-204). Following washing of the immunoprecipitate, the pellet was resuspended in SDS sample buffer and subjected to SDS-PAGE and immunoblotted with affinity purified $GABA_B$R1a-specific antibodies 1713.1 (raised against the peptide acetyl-DVNSRRDILPDYELKLC-amide (a portion of SEQ.ID.NO.:20)) and 1713.2 (raised against the peptide acetyl-CATLHNPTRVKLFEK-amide (a portion of SEQ.ID.NO.:20)).

EXAMPLE 11

Melanophore functional assay

Growth of *Xenopus laevis* melanophores and fibroblasts was performed as described previously (Potenza et al., 1992, Anal. Biochem. 206:315-322). The cells (obtained from Dr. M.R. Lerner, Yale University) were collected by centrifugation at 200xg for 5 min at 4°C, and resuspended at 5 x 10^6 cells per ml in ice cold 70% PBS, pH 7.0. DNA encoding the relevant GPCR was transiently transfected into melanophores by electroporation using a BTX ECM600 electroporator (Genetronics, Inc., San Diego, CA). To monitor the efficiency of transfection, two internal control GPCRs were used independently (pcDNA1amp-cannabinoid 2 and pcDNA3-thromboxane A2; (Lerner, 1994, Trends Neurosci. 17:142-146)). Cells were electroporated using the following settings: capacitance of 325 microfarad, voltage of 450 volts, and resistance of 720 ohms. Following electroporation, cells were mixed with fibroblast-conditioned growth medium and plated onto flat bottom 96 well microtiter plates (NUNC). 24 hrs after the transfection, the media was replaced with fresh fibroblast-conditioned growth media and incubated for an additional day at 27°C prior to assaying for receptor expression. For Gs/Gq-coupling responses (resulting in pigment dispersion), cells were incubated in 100 µl of 70% L-15 media containing 15 mM HEPES, pH 7.3, and melatonin (0.8 nM final concentration) for 1 hr in the dark at room temperature, and then incubated in the presence of melatonin (0.8 nM final concentration) for 1 h in the dark at room temperature to induce pigment aggregation. For Gi-coupled responses (resulting in pigment aggregation), cells were incubated in the presence of 100 µl/well of 70% L-15 media containing 2.5% fibroblast-conditioned growth medium, 2 mM glutamine, 100 µg /ml streptomycin, 100 units/ml penicillin and 15 mM HEPES, pH 7.3, for 30 min in the dark at room temperature to induce pigment dispersion. Absorbance readings at 600 nm were measured using a Bio-Tek Elx800 Microplate reader (ESBE Scientific) before (Ai) and after

(Af) incubation with ligand (GABA; 1.5 hr in the dark at room temperature).

EXAMPLE 12

Stable and transient transfections and determination of cAMP response in HEK293 cells

HG20 and murine $GABA_BR1a$ cDNAs were subcloned into pcDNA3.1 (Invitrogen, San Diego, CA) and used to transfect HEK293 cells. Stably expressing cells were identified after selection in geneticin (0.375 mg/ml) by dot blot analysis. For co-expression experiments, the stable cell lines hgb2-42 (expressing HG20) and rgb1a-50 (expressing murine $GABA_BR1a$) were transiently transfected with murine $GABA_BR1a$ and HG20, respectively, in pcDNA3.1 and cells were assayed for cAMP responses.

Wild-type HEK293 cells, or HEK293 cells stably and transiently expressing HG20 and murine $GABA_BR1a$ receptors were lifted in 1X PBS, 2.5 mM EDTA, counted, pelleted and resuspended at 1.5 x 10^5 cells per 100μl in Krebs-Ringer-Hepes medium (Blakely et al., 1991, Anal. Biochem. 194:302-308), 100 mM Ro 20-1724 (RBI) and incubated at 37°C for 20 min. 100 μl of cells was added to 100 μl of prewarmed (37°C, 10 min) Krebs-Ringer-Hepes medium, 100 mM Ro 20-1724 without or with agonist and/or 10 μM forskolin. Incubations with GABA included 100 μM aminooxyacetic acid (a GABA transaminase inhibitor) to prevent breakdown of GABA and 100 μM nipecotic acid to block GABA uptake. Following a 20 min incubation at 37°C, the assay was terminated by setting the cells on ice and centrifuging at 2,000 rpm for 5 min at 4°C. 175 ml of assay solution was removed and replaced with 175 ml of 0.1 N hydrochloric acid, 0.1 mM calcium chloride and cells were set on ice for 30 min and then stored at -20°C. cAMP determinations were made using a solid phase modification (Maidment et al., 1989, Neurosci. 33:549-557) of the cAMP radioimmunoassay described by Brooker et al. 1979, Adv. Cyclic Nucl. Res. 10:1-33) and previously reported in Clark et al., 1998, Mol.

Endocrinol. 12:193-206). Immulon II removawells (Dynatech; Chantilly, VA) were coated overnight with 100 µl of protein G (1mg/ml in 0.1M $NaHCO_3$, pH 9.0) at 4°C. Prior to use, protein G-coated plates were rinsed with PBS-gelatin-Tween (phosphate buffered saline containing 0.1% gelatin, 0.2% Tween-20) 3 times quickly, and then once for 30 minutes. Following the rinse with PBS-gelatin-Tween, the RIA was set up by adding 100 µl 50 mM sodium acetate, pH 4.75, cAMP standards or aliquots from treated cells, 5,000-7,000 cpm ^{125}I-succinyl cAMP, and 25 µl of a sheep antibody to cAMP diluted in 50 mM sodium acetate, pH 4.75 (Atto instruments; dilution of stock to 2.5×10^{-5}, determined empirically) to the plates in a final volume of 175 µl. Plates were incubated 2 hr at 37°C or overnight at 4°C, rinsed 3 times with sodium acetate buffer, blotted dry, and then individual wells were broken off and bound radioactivity was determined in a gamma counter.

EXAMPLE 13

In situ hybridization for co-localization experiments

Preparation of rat brain sections, prehybridization and hybridization of rat brain slices was performed as described previously (Bradley et al., 1992, J. Neurosci. 12:2288-2302; http://intramural.nimh.nih.gov/lcmr/snge/Protocol.html). Adjacent coronal rat brain sections were hybridized with labeled antisense and sense riboprobes directed against HG20 (GenBank accession number AF058795) or murine $GABA_BR1a$.

HG20 probes were generated by amplification of HG20 with JC216 (T3 promotor/primer and bases 1172-1191) paired with JC217 (T7 promotor/primer and bases 1609-1626) or with JC218 (T3 promotor/primer and bases 2386-2405) paired with JC219 (T7 promotor/primer and bases 2776-2793):
(JC216: cgcgcaattaaccctcactaaaggACAACAGCAAACGTTCAGGC (SEQ.ID.NO.:28);

JC217: gcgcgtaatacg actcactatagggCATGCCTATGATGGTGAG (SEQ.ID.NO.:29);
JC218: cgcgcaattaaccctcactaaagg CTGAGGACAAACCCTGACGC (SEQ.ID.NO.:30);
JC219: gcgcgtaatacgactcactatagggGATGTC TTCTATGGGGTC; (SEQ.ID.NO.:31)).

Murine GABA$_B$R1a probes were generated by amplification of murine GABA$_B$R1a with JC160 (T3 promotor/primer and bases 631-648) paired with JC161 (T7 promotor/primer and bases 1024-1041): (JC160: cgcgcaattaaccctcactaaaggAAGCTTATCCACCACGAC (SEQ.ID.NO.:32);
JC161:gcgcgtaa tacgactcactatagggAGCTGGATCCGAGAAGAA (SEQ.ID.NO.:33)).

For colocalization experiments, murine GABA$_B$R1a probes were labeled with digoxigenin-UTP and detected using a peroxidase-conjugated antibody to digoxigenin and TSA amplification involving biotinyl tyramide and subsequent detection with streptavidin-conjugated fluorescein. HG20 probes were radiolabelled (http://intramural.nimh.nih.gov/lcmr/snge/Protocol.html). For individual hybridizations, murine GABA$_B$R1a and HG20 riboprobes were radiolabeled with ^{35}S-UTP and detected as described previously (Bradley et al., 1992, J. Neurosci. 12:2288-2302; http://intramural.nimh.nih. gov/lcmr/snge/Protocol.html). Brain slices were either hybridized with individual radiolabelled probes or, for colocalization studies, simultaneously with probes to both murine GABA$_B$R1a and HG20 receptors. Detection of the radiolabeled HG20 probe was performed after detection of the digoxigenin-labeled rgb1 probe on the same brain slices.

EXAMPLE 14

Construction of N-terminal and C-terminal fragments of murine $GABA_BR1a$

The N-terminal fragment of murine $GABA_BR1a$, comprising amino acid positions 1-625, was generated by PCR. The coding sequence of the N-terminal fragment was amplified by using primer pairs: NFP-CJ7843F139 (5'- ACC ACT GCT AGC ACC GCC ATG CTG CTG CTG CTG CTT CTG C -3'; SEQ.IS.NO.:34) and NRP-CJ7844 (3'- GG GTG CGA GCA ATA TAG GTC TTA AGG GTC GGC CGC CGG CGT CAC CA -5'; ; SEQ.IS.NO.:35). Similarly, the C-terminal fragment, amino acid positions 588-942, was generated by PCR using primer pairs: CFP-CJ7845 (5'- ACC ACT GCT AGC ACC GCC ATG CAG AAA CTC TTT ATC TCC GTC TCA GTT CTC TCC AGC-3'; ; SEQ.IS.NO.:36) and CRP-CJ7846 (3'- CAG CTC ATG TAA ACG AAA TGT TCA CTC GCC GGC CGC CGG CGT CAC CA-5'; ; SEQ.IS.NO.:37). PCR reactions were carried out using the Advantage-HF PCR kit (Clontech, Paolo Alto, CA) with 0.2 ng of murine $GABA_BR1a$ DNA as the template, and 10 μM of each primer according to manufacturer instructions. The PCR conditions were as follows: precycle denaturation at 94°C for 1 min, and then 35 cycles at 94°C (15 s), annealing and extension at 72°C (3 min), followed by a final extension for 3 min at 72°C. The PCR products, N-gb 1a and C-gb 1a DNA, flanked by Nhe1 and Not1 sites, were digested and subcloned into the Nhe1/Not1 site of pcDNA3.1 (Invitrogen, San Diego, Ca).

EXAMPLE 15

Cell culture and preparation of membrane fractions for binding experiments using N-terminal and C-terminal $GABA_BR1a$ fragments

COS-7 cells (ATCC) were cultured in DMEM, 10% bovine serum, 25 mM HEPES, and antibiotics and transiently transfected with murine gb1a/pcDNA3.1 (encoding full-length $GABA_BR1a$), N-gb

1a/pcDNA3.1 (encoding the N-terminal fragment of $GABA_BR1a$; see Example 14) or C-gb 1a/pcDNA3.1 (encoding the C-terminal fragment of $GABA_BR1a$; see Example 14) using Lipofectamine reagent (Gibco BRL) following the conditions recommended by the manufacturer. At 48 h post-transfection, P2 membrane fractions were prepared at 4°C as follows: Cells were washed twice with cold PBS, collected by centrifugation at 100xg for 7 min, and resuspended in 10 ml of buffer A: 5 mM Tris-HCl, 2 mM EDTA containing (1X) protease inhibitor cocktail Complete® tablets (Boehringer Mannheim), pH 7.4 at 4°C. Cells were disrupted by polytron homogenization, centrifuged at 100xg for 7 min to pellet unbroken cells and nuclei, and the supernatant collected. The resulting pellet was homogenized a second time in 10 ml of buffer A, centrifuged as described above and supernatant fractions saved. The pooled S1 supernatant was centrifuged at high speed (27,000xg for 20 min) and the pellet was washed once with buffer A, centrifuged (27,000xg for 20 min), resuspended in buffer A to make the P2 membrane fraction, and stored at -80°C. Protein content was determined using the Bio-Rad Protein Assay Kit according to manufacturer instructions.

EXAMPLE 16

In vitro transcription/translation of $GABA_BR1a$ and N-terminal and C-terminal fragments

In vitro transcription coupled translation reactions were performed in the presence of [^{35}S]-methionine in the TNT Coupled Reticulocyte Lysate system (Promega, WI) using the pcDNA3.1 plasmid containing the full-length $GABA_BR1a$, N-gb1a, and C-gb1a DNAs. Translation products were analysed by electrophoresis on 8-16% Tris-Glycine gradient gels (Novex pre-cast gel system) under denaturing and reducing conditions. Gels were fixed, treated with enlightening fluid (NEN), dried and exposed to Kodak X-AR film at -70°C for 4 to 24 h. Analysis of the results of these *in vitro* transcription coupled translation reactions confirmed that the constructs whose production is described in Example 14 directed the

expression of the appropriate $GABA_BR1a$ fragments (see Figure 17A).

EXAMPLE 17

Immunoblot analysis for experiments with N-terminal and C-terminal fragments of $GABA_BR1a$

The expression of full-length and N-terminal and C-terminal $GABA_BR1a$ fragments *in vivo* was confirmed by immunoblot analysis. Membranes were solubilized in SDS sample buffer consisting of 50 mM Tris-HCl pH 6.5, 10% SDS, 10% glycerol, and 0.003% bromophenol blue with 10% 2-mercaptoethanol and separated on SDS-PAGE. The full-length receptor and N-terminal fragment were detected using affinity purified rabbit $GABA_BR1a$ polyclonal antibody 1713.1 (acetyl-DVNSRRDILPDYELKLC-amide; a portion of SEQ.ID.NO.:20) and 1713.2 (acetyl-CATLHNPTRVKLFEK-amide; a portion of SEQ.ID.NO.:20) (Quality Control Biochemicals (Hopkinton, MA). The C-terminal fragment was detected using a $GABA_BR1a$ antibody raised against the C-terminal tail of the receptor (acetyl-PSEPPDRLSCDGSRVHLLYK-amide; SEQ.ID.NO.:20) (Chemicon Int., Inc., Canada).

EXAMPLE 18

Receptor filter-binding assays for experiments with N-terminal and C-terminal fragments of $GABA_BR1a$

Competition of $[^{125}I]$ CGP71872 binding experiments were performed with ~7 μg P2 membrane protein and increasing concentrations of cold ligand (10^{-12}-10^{-3} M). The concentration of radioligand used in the competition assays was 1 nM (final). Each concentration was examined in duplicate and incubated for 2 hr at 22°C in the dark in a total volume of 250 μL binding buffer: 50 mM Tris-HCl, 2.5 mM $CaCl_2$ (pH 7.4) with (1X) protease inhibitor cocktail Complete® tablets. Bound ligand was isolated by rapid filtration through a Brandel

96 well cell harvester using Whatman GF/B filters. Data were analysed by nonlinear least-squares regression using the computer-fitting program GraphPad Prism version 2.01 (San Diego).

EXAMPLE 19

Photoaffinity labeling for experiments with N-terminal and C-terminal fragments of $GABA_BR1a$

P2 membranes were resuspended in binding buffer, and incubated in the dark with 1 nM final concentration [^{125}I]CGP71872 (2200 Ci/mmol) in a final volume of 1 ml for 2 h at 22°C. The membranes were centrifuged at 27, 000xg for 10 min and the pellet was washed in ice-cold binding buffer, centrifuged at 27, 000xg for 20 min and resuspended in 1 ml of ice-cold binding buffer and exposed on ice 2 inches from 360 nm ultraviolet light for 10 min. Photolabeled membranes were washed and membranes pelleted by centrifugation and solubilized in sample buffer (50 mM Tris-HCl pH 6.5, 10% SDS, 10% glycerol, and 0.003% bromophenol blue with 10% 2-mercaptoethanol). Samples were electrophoresed on precast NOVEX 10% Tris-glycine gels, fixed, dried, and exposed to Kodak XAR film with an intensifying screen at –70°C.

EXAMPLE 20

Construction of the FLAG epitope-tagged HG20 and detection of expression in vitro and in COS-1 cells

The FLAG epitope-tagged HG20 receptor subunit was constructed by PCR using a sense primer encoding a modified influenza hemaglutinin signal sequence (MKTIIALSYIFCLVFA; a portion of SEQ.ID.NO.:17) (Jou et al., 1980, Cell 19:683-696) followed by an antigenic FLAG epitope (DYKDDDDK; a portion of SEQ.ID.NO.:17) and DNA encoding amino acids 52-63 of HG20 and an antisense primer encoding amino acids 930-941 of the HG20 in a high-fidelity PCR reaction

with HG20/pCR 3.1 as a template. HG20/pCR 3.1 is a plasmid that contains full-length HG20 (SEQ.ID.NO.:2) cloned into pCR3.1. The nucleotide sequences of the sense and antisense primers are: sense: 5'-GCC GCT AGC GCC ACC ATG AAG ACG ATC ATC GCC CTG AGC TAC ATC TTC TGC CTG GTA TTC GCC GAC TAC AAG GAC GAT GAT GAC AAG AGC AGC CCG CCG CTC TCC ATC ATG GGC CTC ATG CCG CTC-3', (SEQ.ID.NO.:38); antisense: 5'-GCC TCT AGA TTA CAG GCC CGA GAC CAT GAC TCG GAA GGA GGG TGG CAC-3'. (SEQ.ID.NO.:39). The PCR conditions were: precycle denaturation at 94°C for 1 min, 94°C for 30 sec, annealing and extension at 72°C for 4 min for 25 cycles, followed by a 7 min extension at 72°C . The PCR product, SF-HG20 DNA, flanked by NheI and XbaI sites was subcloned into the NheI/XbaI site of pcDNA3.1 (Invitrogen, San Diego, Ca) to give rise to the expression construct SF-HG20/pcDNA3.1. The sequence of this construct was verified on both strands.

The SF-HG20 receptor was expressed in an *in vitro* coupled transcription/translation reaction using the TNT Coupled Reticulocyte Lysate system (Promega, WI) in the presence of [^{35}S]methionine according to the manufacturer instructions. Radiolabeled proteins were analyzed by electrophoresis on 8-16% Tris-Glycine gradient gels (Novex pre-cast gel system) under denaturing and reducing conditions. Gels were fixed and treated with Enlightening fluid (NEN), dried and exposed to Kodak X-AR film at -70°C.

COS-1 cells (ATCC, CRL 1650) were cultured in DMEM, 10% bovine serum, 25 mM HEPES, pH 7.4, and 10 units/mL penicillin- 10 µg/mL streptomycin. Transient transfection of COS-1 cells with SF-HG20/pcDNA 3.1 was carried out using Lipofectamine reagent (Gibco BRL) following the conditions recommended by the manufacturer. At 48 h post-transfection, crude membranes were prepared and receptors were solubilized with digitonin and immunoprecipitated with anti-FLAG M2 affinity gel resin (IBI) under previously described conditions (Ng et al., 1993). The immunoprecipitate was washed and solubilized in SDS sample buffer, sonicated, electrophoresed, and blotted on to nitrocellulose membrane as described (Ng et al., 1993). The FLAG-tagged HG20

receptor was detected using an anti-FLAG antibody (Santa Cruz Biotech., Inc.) by following a chemilumescence protocol of the manufacturer (NEN).

EXAMPLE 21

Kir channel activity in *Xenopus* oocytes

With the following modifications, *Xenopus* oocytes were isolated as described (Hébert et al., 1994, Proc. R. Soc. Lond. B 256:253-261) from live frogs supplied by Boreal, Inc. After a brief (10 min) hypertonic shock with 125 mM potassium phosphate pH 6.5, oocytes were allowed to recover in Barth's solution for 1-2 hr. cDNA constructs for human Kir 3.1, Kir 3.2 channel isoforms (generous gifts from Dr. Hubert Van Tol, University of Toronto), and Giα1 (a generous gift of Dr. Maureen Linder, Washington University) were linearized by restriction enzymes and purified using Geneclean (Bio 101). Murine $GABA_BR1a$ or FLAG-HG20 clones were subcloned into pT7TS (a generous gift of Dr. Paul Krieg, University of Texas) before linearization and transcription. Capped cRNA was made using T7 RNA polymerase and the mMessage mMachine (Ambion). Individual oocytes were injected with 5-10 ng (in 25-50 nL) of Kir3.1 and Kir3.2 constructs with mRNAs for murine $GABA_BR1a$ or FLAG-HG20 and in combination with Giα1 as well. Kir currents were also evaluated in ooctyes co-injected with Kir3.1, Kir3.2, murine $GABA_BR1a$ and FLAG-HG20 mRNAs. Currents were recorded after 48 hr. Standard recording solution was KD-98, 98 mM KCl, 1 mM $MgCl_2$, 5 mM K-HEPES, pH 7.5, unless otherwise stated. Microelectrodes were filled with 3 M KCl and had resistances of 1-3 MW and 0.1-0.5 MW for voltage and current electrodes, respectively. In addition, current electrodes were backfilled with 1% agarose (in 3M KCl) to prevent leakage as described (Hébert et al., 1994, Proc. R. Soc. Lond. B 256:253-261). Recordings were made at room temperature using a Geneclamp 500 amplifier (Axon Instruments). Oocytes were voltage clamped and perfused continuously with different recording solutions. Currents were evoked by 500 msec voltage commands from a holding potential of -10

mV, delivered in 20 mV increments from -140 to 60 mV to test for inward rectifying potassium currents. Data were recorded at a holding potential of -80 mV and drugs were added to the bath with a fast perfusion system. Data collection and analysis were performed using pCLAMP v6.0 (Axon Instruments) and Origin v4.0 (MicroCal) software. For subtraction of endogenous and leak currents, records were obtained in ND-96, 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM Na-HEPES and these were subtracted from recordings in KD-98 before further analysis.

EXAMPLE 22

Radiation Hybrid mapping of HG20

Radiation hybrid analysis assigned the HG20 gene to chromosome 9, placing it 4.81 cR from the WI-8684 marker on the GeneBridge 4 panel of 93 RH clones of the whole human genome. Searching of the OMIM database with D9S176 and D9S287 markers proximal to the HG20 gene revealed it to map proximal to the hereditary sensory neuropathy type 1 (HSN-1) locus, a ~8 cM region flanked by D9S176 and 9S318 (Nicholson et al., 1996, Nature Genetics 13, 101-104) (Figure 20). HSN-1 is the most common form of a group of degenerative disorders of sensory neurons characterized by a progressive degeneration of dorsal root ganglion and motor neurons that lead to distal sensory loss, distal muscle wasting and weakness, and neural deafness, among a number of other neuronally related deficits (Nicholson et al., 1996, Nature Genetics 13, 101-104). FCMD (Fukuyama congenital muscular dystrophy) and DYS (dysautonomia, another type of HSN) also map to this area. Candidate gene(s) in these disorders are likely critical to the development, survival, and differentiation of neurons.

A human BAC library was screened using the EcoRI fragment containing the full-length HG20 DNA, and end-sequencing was performed on BAC clones designated 6D18, 168K19, 486B24, and 764N4. The primer pair: ngf1t7+ (5'-AAC AGT CAA AAC CCA CCC AG-3'; SEQ.ID.NO.:40) and ngf1t7- (5'-AAC AGT TTC CAG CTG TGC CT-3';

SEQ.ID.NO.:41) were identified for radiation hybrid mapping of the HG20 gene on the GENEBRIDGE 4 panel. BAC library screening and radiation hybrid mapping were performed by Research Genetics (Huntsville, AL).

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED:

1. An isolated DNA molecule encoding an HG20 polypeptide comprising an amino acid sequence selected from the group consisting of:

SEQ.ID.NO.:2;
Positions 9-941 of SEQ.ID.NO.:2;
Positions 35-941 of SEQ.ID.NO.:2;
Positions 36-941 of SEQ.ID.NO.:2;
Positions 38-941 of SEQ.ID.NO.:2;
Positions 39-941 of SEQ.ID.NO.:2;
Positions 42-941 of SEQ.ID.NO.:2;
Positions 44-941 of SEQ.ID.NO.:2;
Positions 46-941 of SEQ.ID.NO.:2;
Positions 52-941 of SEQ.ID.NO.:2; and
Positions 57-941 of SEQ.ID.NO.:2.

2. The isolated DNA molecule of claim 1 comprising a nucleotide sequence selected from the group consisting of:

SEQ.ID.NO.:1;
Positions 293-3,115 of SEQ.ID.NO.:1;
Positions 317-3,115 of SEQ.ID.NO.:1;
Positions 395-3,115 of SEQ.ID.NO.:1;
Positions 398-3,115 of SEQ.ID.NO.:1;
Positions 404-3,115 of SEQ.ID.NO.:1;
Positions 407-3,115 of SEQ.ID.NO.:1;
Positions 416-3,115 of SEQ.ID.NO.:1;
Positions 422-3,115 of SEQ.ID.NO.:1;
Positions 428-3,115 of SEQ.ID.NO.:1;
Positions 446-3,115 of SEQ.ID.NO.:1; and
Positions 461-3,115 of SEQ.ID.NO.:1.

3. An isolated DNA molecule that hybridizes under stringent conditions to the DNA molecule of claim 2.

4. An expression vector comprising the DNA of claim 1.

5. A recombinant host cell comprising the expression vector of claim 4.

6. The recombinant cell of claim 5 further comprising an expression vector comprising DNA encoding a protein selected from the group consisting of:

7. A protein, substantially free from other proteins, comprising an HG20 protein having an amino acid sequence selected from the group consisting of:

SEQ.ID.NO.:2;
Positions 9-941 of SEQ.ID.NO.:2;
Positions 35-941 of SEQ.ID.NO.:2;
Positions 36-941 of SEQ.ID.NO.:2;
Positions 38-941 of SEQ.ID.NO.:2;
Positions 39-941 of SEQ.ID.NO.:2;
Positions 42-941 of SEQ.ID.NO.:2;
Positions 44-941 of SEQ.ID.NO.:2;
Positions 46-941 of SEQ.ID.NO.:2;
Positions 52-941 of SEQ.ID.NO.:2; and
Positions 57-941 of SEQ.ID.NO.:2.

8. A heterodimer comprising the protein of claim 7 and a G-protein coupled receptor protein where the heterodimer is substantially free from other proteins.

9. The heterodimer of claim 8 where the heterodimer is held together by N-terminal Sushi repeats, C-terminal alpha-helical

interacting domains, coiled-coil domains, transmembrane interactions, or disulfide bonds.

10. A polypeptide comprising a coiled-coil domain from a first $GABA_B$ receptor subunit and no other contiguous amino acid sequences longer than 5 amino acids from the first $GABA_B$ receptor subunit where the coiled-coil domain is present in the C-terminus of the $GABA_B$ receptor subunit and mediates heterodimerization of the first $GABA_B$ receptor subunit with a second $GABA_B$ receptor subunit.

11. The polypeptide of claim 10 where the coiled-coil domain is selected from the group consisting of: positions 756-829 of SEQ.ID.NO.:2; positions 779-814 of SEQ.ID.NO.:2; positions 886-949 of SEQ.ID.NO.:21; and positions 889-934 of SEQ.ID.NO.:21.

12. An isolated DNA molecule encoding a $GABA_BR1a$ polypeptide comprising the amino acid sequence SEQ.ID.NO.:20.

13. A protein, substantially free from other proteins, comprising a $GABA_BR1a$ protein having the amino acid sequence SEQ.ID.NO.:20.

14. A method for determining whether a substance binds $GABA_B$ receptors and is thus a potential agonist or antagonist of the $GABA_B$ receptor that comprises:

(a) providing cells c1`omprising an expression vector encoding HG20 and an expression vector encoding $GABA_BR1a$ or $GABA_BR1b$;

(b) culturing the cells under conditions such that HG20 and $GABA_BR1a$ or $GABA_BR1b$ are expressed and heterodimers of HG20 and $GABA_BR1a$ or $GABA_BR1b$ are formed;

(c) exposing the cells to a labeled ligand of $GABA_B$ receptors in the presence and in the absence of the substance;

(d) measuring the binding of the labeled ligand to the heterodimers of HG20 and $GABA_BR1a$ or $GABA_BR1b$;

where if the amount of binding of the labeled ligand is less in the presence of the substance than in the absence of the substance, then the substance is a potential agonist or antagonist of $GABA_B$ receptors.

15. A method of identifying agonists and antagonists of HG20 comprising:

(a) providing test cells by transfecting cells with:

(1) an expression vector that directs the expression of HG20 in the cells; and

(2) an expression vector that directs the expression of $GABA_BR1a$ or $GABA_BR1b$ in the cells;

(b) exposing the test cells to a substance that is suspected of being an agonist of the $GABA_B$ receptor;

(c) measuring the amount of a functional response of the test cells that have been exposed to the substance;

(d) comparing the amount of the functional response exhibited by the test cells with the amount of the functional response exhibited by control cells;

wherein if the amount of the functional response exhibited by the test cells differs from the amount of the functional response exhibited by the control cells, the substance is an agonist or antagonist of the $GABA_B$ receptor;

where the control cells are cells that have not been transfected with HG20 and $GABA_BR1a$ or $GABA_BR1b$ but have been exposed to the substance or are test cells that have not been exposed to the substance.

16. A method of producing functional $GABA_B$ receptors in cells comprising:

(a) transfecting cells with:

(1) an expression vector that directs the expression of HG20 in the cells; and

(2) an expression vector that directs the expression of $GABA_BR1a$ or $GABA_BR1b$ in the cells;

(b) culturing the cells under conditions such that heterodimers of HG20 and $GABA_BR1a$ or $GABA_BR1b$ are formed where the heterodimers constitue functional $GABA_B$ receptors.

17. An antibody that binds specifically to HG20 where HG20 has an amino acid sequence selected from the group consisting of:

SEQ.ID.NO.:2;
Positions 9-941 of SEQ.ID.NO.:2;
Positions 35-941 of SEQ.ID.NO.:2;
Positions 36-941 of SEQ.ID.NO.:2;
Positions 38-941 of SEQ.ID.NO.:2;
Positions 39-941 of SEQ.ID.NO.:2;
Positions 42-941 of SEQ.ID.NO.:2;
Positions 44-941 of SEQ.ID.NO.:2;
Positions 46-941 of SEQ.ID.NO.:2;
Positions 52-941 of SEQ.ID.NO.:2; and
Positions 57-941 of SEQ.ID.NO.:2.

18. A method of expressing a truncated version of HG20 protein comprising:

(a) transfecting a host cell with a expression vector that encodes an HG20 protein that has been truncated at the amino terminus;

(b) culturing the transfected cells of step (a) under conditions such that the truncated HG20 protein is expressed.

19. A chimeric HG20 protein having an amino acid sequence of HG20 selected from the group consisting of:

Positions 51-941 of SEQ.ID.NO.:2;
Positions 52-941 of SEQ.ID.NO.:2;
Positions 53-941 of SEQ.ID.NO.:2;
Positions 54-941 of SEQ.ID.NO.:2;
Positions 55-941 of SEQ.ID.NO.:2;

Positions 56-941 of SEQ.ID.NO.:2;
Positions 57-941 of SEQ.ID.NO.:2; and
Positions 58-941 of SEQ.ID.NO.:2;

covalently linked at the N-terminus with a non-HG20 amino acid sequence.

1 CCGCCCTCCC CCGGCCGAGC TCCAGGGCTG CCGCCTAGCA GCTCCCGGCG
51 GGAGAGCGGT TCAGAGCTCG CTCCCACCCC TTCCCGGCGT GATTGATCCG
101 TCACGGGCGC CTCCGCTGCC GCCGCCGCCG CCGCGGCCGT TCTGAGCCGA
151 GCCGGAACCC TAGCCCGAGA CGGAGCCGGG GCCCGGGCCG GCGCCATTGC
201 GCGGGCGCCG CGGGAAGACC TTGGCGCGGG GCGGCGGGCC GGGCCAGGCC
251 ATGCGGGCCG AGTGAGCCGG CGCCCGCAGC CCGCGGCGCG GCATGGCTTC
301 CCCGCGGAGC TCCGGGCAGC CCGGGCCGCC GCCGCCGCCG CCACCGCCGC
351 CCGCGCGCCT GCTACTGCTA CTGCTGCTGC CGCTGCTGCT GCCTCTGGCG
401 CCCGGGGCCT GGGGCTGGGC GCGGGGCGCC CCCCGGCCGC CGCCCAGCAG
451 CCCGCCGCTC TCCATCATGG GCCTCATGCC GCTCACCAAG GAGGTGGCCA
501 AGGGCAGCAT CGGGCGCGGT GTGCTCCCCG CCGTGGAACT GGCCATCGAG
551 CAGATCCGCA ACGAGTCACT CCTGCGCCCC TACTTCCTCG ACCTGCGGCT
601 CTATGACACG GAGTGCGACA ACGCAAAAGG GTTGAAAGCC TTCTACGATG
651 CAATAAAATA CGGGCCGAAC CACTTGATGG TGTTTGGAGG CGTCTGTCCA
701 TCCGTCACAT CCATCATTGC AGAGTCCCTC CAAGGCTGGA ATCTGGTGCA
751 GCTTTCTTTT GCTGCAACCA CGCCTGTTCT AGCCGATAAG AAAAAATACC
801 CTTATTTCTT TCGGACCGTC CCATCAGACA ATGCGGTGAA TCCAGCCATT
851 CTGAAGTTGC TCAAGCACTA CCAGTGGAAG CGCGTGGGCA CGCTGACGCA
901 AGACGTTCAG AGGTTCTCTG AGGTGCGGAA TGACCTGACT GGAGTTCTGT
951 ATGGCGAGGA CATTGAGATT TCAGACACCG AGAGCTTCTC CAACGATCCC
1001 TGTACCAGTG TCAAAAAGCT GAAGGGGAAT GATGTGCGGA TCATCCTTGG
1051 CCAGTTTGAC CAGAATATGG CAGCAAAAGT GTTCTGTTGT GCATACGAGG
1101 AGAACATGTA TGGTAGTAAA TATCAGTGGA TCATTCCGGG CTGGTACGAG
1151 CCTTCTTGGT GGGAGCAGGT GCACACGGAA GCCAACTCAT CCCGCTGCCT
1201 CCGGAAGAAT CTGCTTGCTG CCATGGAGGG CTACATTGGC GTGGATTTCG
1251 AGCCCCTGAG CTCCAAGCAG ATCAAGACCA TCTCAGGAAA GACTCCACAG
1301 CAGTATGAGA GAGAGTACAA CAACAAGCGG TCAGGCGTGG GGCCCAGCAA
1351 GTTCCACGGG TACGCCTACG ATGGCATCTG GGTCATCGCC AAGACACTGC
1401 AGAGGGCCAT GGAGACACTG CATGCCAGCA GCCGGCACCA GCGGATCCAG
1451 GACTTCAACT ACACGGACCA CACGCTGGGC AGGATCATCC TCAATGCCAT
1501 GAACGAGACC AACTTCTTCG GGGTCACGGG TCAAGTTGTA TTCCGGAATG
1551 GGGAGAGAAT GGGGACCATT AAATTTACTC AATTTCAAGA CAGCAGGGAG
1601 GTGAAGGTGG GAGAGTACAA CGCTGTGGCC GACACACTGG AGATCATCAA
1651 TGACACCATC AGGTTCCAAG GATCCGAACC ACCAAAAGAC AAGACCATCA
1701 TCCTGGAGCA GCTGCGGAAG ATCTCCCTAC CTCTCTACAG CATCCTCTCT
1751 GCCCTCACCA TCCTCGGGAT GATCATGGCC AGTGCTTTTC TCTTCTTCAA
1801 CATCAAGAAC CGGAATCAGA AGCTCATAAA GATGTCGAGT CCATACATGA
1851 ACAACCTTAT CATCCTTGGA GGGATGCTCT CCTATGCTTC CATATTTCTC
1901 TTTGGCCTTG ATGGATCCTT TGTCTCTGAA AAGACCTTTG AAACACTTTG
1951 CACCGTCAGG ACCTGGATTC TCACCGTGGG CTACACGACC GCTTTTGGGG
2001 CCATGTTTGC AAAGACCTGG AGAGTCCACG CCATCTTCAA AAATGTGAAA
2051 ATGAAGAAGA AGATCATCAA GGACCAGAAA CTGCTTGTGA TCGTGGGGGG
2101 CATGCTGCTG ATCGACCTGT GTATCCTGAT CTGCTGGCAG GCTGTGGACC
2151 CCCTGCGAAG GACAGTGGAG AAGTACAGCA TGGAGCCGGA CCCAGCAGGA

FIG.1A

2201 CGGGATATCT CCATCCGCCC TCTCCTGGAG CACTGTGAGA ACACCCATAT
2251 GACCATCTGG CTTGGCATCG TCTATGCCTA CAAGGGACTT CTCATGTTGT
2301 TCGGTTGTTT CTTAGCTTGG GAGACCCGCA ACGTCAGCAT CCCCGCACTC
2351 AACGACAGCA AGTACATCGG GATGAGTGTC TACAACGTGG GGATCATGTG
2401 CATCATCGGG GCCGCTGTCT CCTTCCTGAC CCGGGACCAG CCCAATGTGC
2451 AGTTCTGCAT CGTGGCTCTG GTCATCATCT TCTGCAGCAC CATCACCCTC
2501 TGCCTGGTAT TCGTGCCGAA GCTCATCACC CTGAGAACAA ACCCAGATGC
2551 AGCAACGCAG AACAGGCGAT TCCAGTTCAC TCAGAATCAG AAGAAAGAAG
2601 ATTCTAAAAC GTCCACCTCG GTCACCAGTG TGAACCAAGC CAGCACATCC
2651 CGCCTGGAGG GCCTACAGTC AGAAAACCAT CGCCTGCGAA TGAAGATCAC
2701 AGAGCTGGAT AAAGACTTGG AAGAGGTCAC CATGCAGCTG CAGGACACAC
2751 CAGAAAAGAC CACCTACATT AAACAGAACC ACTACCAAGA GCTCAATGAC
2801 ATCCTCAACC TGGGAAACTT CACTGAGAGC ACAGATGGAG GAAAGGCCAT
2851 TTTAAAAAAT CACCTCGATC AAAATCCCCA GCTACAGTGG AACACAACAG
2901 AGCCCTCTCG AACATGCAAA GATCCTATAG AAGATATAAA CTCTCCAGAA
2951 CACATCCAGC GTCGGCTGTC CCTCCAGCTC CCCATCCTCC ACCACGCCTA
3001 CCTCCCATCC ATCGGAGGCG TGGACGCCAG CTGTGTCAGC CCCTGCGTCA
3051 GCCCCACCGC CAGCCCCCGC CACAGACATG TGCCACCCTC CTTCCGAGTC
3101 ATGGTCTCGG GCCTGTAAGG GTGGGGGGCC TGGGCCCGGG GCCTCCCCCG
3151 TGACAGAACC ACACTGGGCA GAGGGGTCTG CTGCAGAAAC ACTGTCGGCT
3201 CTGGCTGCGG AGAAGCTGGG CACCATGGCT GGCCTCTCAG GACCACTCGG
3251 ATGGCACTCA GGTGGACAGG ACGGGGCAGG GGGAGACTTG GCACCTGACC
3301 TCGAGCCTTA TTTGTGAAGT CCTTATTTCT TCACAAAGAA GAGGAACGGA
3351 AATGGGACGT CTTCCTTAAC ATCTGCAAAC AAGGAGGCGC TGGGATATCR
3401 AATTCCACCA CACTGGCGGC CCGCGCTTGS TCSTAATCAT GGTCATAACT
3451 GTTTCCTGTG TTGAAATTGT TATCCGCTCC

FIG.1B

1 MASPRSSGQP GPPPPPPPPP ARLLLLLLLP LLLPLAPGAW GWARGAPRPP

51 PSSPPLSIMG LMPLTKEVAK GSIGRGVLPA VELAIEQIRN ESLLRPYFLD

101 LRLYDTECDN AKGLKAFYDA IKYGPNHLMV FGGVCPSVTS IIAESLQGWN

151 LVQLSFAATT PVLADKKKYP YFFRTVPSDN AVNPAILKLL KHYQWKRVGT

201 LTQDVQRFSE VRNDLTGVLY GEDIEISDTE SFSNDPCTSV KKLKGNDVRI

251 ILGQFDQNMA AKVFCCAYEE NMYGSKYQWI IPGWYEPSWW EQVHTEANSS

301 RCLRKNLLAA MEGYIGVDFE PLSSKQIKTI SGKTPQQYER EYNNKRSGVG

351 PSKFHGYAYD GIWVIAKTLQ RAMETLHASS RHQRIQDFNY TDHTLGRIIL

401 NAMNETNFFG VTGQVVFRNG ERMGTIKFTQ FQDSREVKVG EYNAVADTLE

451 IINDTIRFQG SEPPKDKTII LEQLRKISLP LYSILSALTI LGMIMASAFL

501 FFNIKNRNQK LIKMSSPYMN NLIILGGMLS YASIFLFGLD GSFVSEKTFE

551 TLCTVRTWIL TVGYTTAFGA MFAKTWRVHA IFKNVKMKKK IIKDQKLLVI

601 VGGMLLIDLC ILICWQAVDP LRRTVEKYSM EPDPAGRDIS IRPLLEHCEN

651 THMTIWLGIV YAYKGLLMLF GCFLAWETRN VSIPALNDSK YIGMSVYNVG

701 IMCIIGAAVS FLTRDQPNVQ FCIVALVIIF CSTITLCLVF VPKLITLRTN

751 PDAATQNRRF QFTQNQKKED SKTSTSVTSV NQASTSRLEG LQSENHRLRM

801 KITELDKDLE EVTMQLQDTP EKTTYIKQNH YQELNDILNL GNFTESTDGG

851 KAILKNHLDQ NPQLQWNTTE PSRTCKDPIE DINSPEHIQR RLSLQLPILH

901 HAYLPSIGGV DASCVSPCVS PTASPRHRHV PPSFRVMVSG L

FIG.2

Sequence: LPLLLPLAPGAWG-WARGAPRPPPSSPPLSIMGLMPLTKEVAKGSIGRGVLPAVELAIEQIRNE
|(signal) |(mature peptide)
29 42

Other entries above 3.50

Score 11.1 at residue 39

Sequence: LLLLPLLLPLAPG-AWGWARGAPRPPPSSPPLSIMGLMPLTKEVAKGSIGRGVLPAVELAIEQI
|(signal) |(mature peptide)
26 39

Score 8.6 at residue 38

Sequence: LLLLLPLLLPLAP-GAWGWARGAPRPPPSSPPLSIMGLMPLTKEVAKGSIGRGVLPAVELAIEQ
|(signal) | (mature peptide)
25 38

Score 8.1 at residue 35

Sequence: RLILLLLLPLLLP-LAPGAWGWARGAPRPPPSSPPLSIMGLMPLTKEVAKGSIGRGVLPAVELA
|(signal) |(mature peptide)
22 35

Score 7.9 at residue 36

Sequence: LILLLLLPLLLPL-APGAWGWARGAPRPPPSSPPLSIMGLMPLTKEVAKGSIGRGVLPAVELAI
|(signal) |(mature peptide)
23 36

Score 6.2 at residue 9

Sequence: -QPGRPPPPPPPPARLILLLLLPLLLPLAPGAWGWARGAPRPPPSSPPLSI
|(signal) |(mature peptide)
-4 9

Score 5.7 at residue 46

Sequence: LPLAPGAWGWARG-APRPPPSSPPLSIMGLMPLTKEVAKGSIGRGVLPAVELAIEQIRNESLLR
|(signal) |(mature peptide)
33 46

Score 5.6 at residue 747

Sequence: ITLCLVFVPKLIT-LRTNPDAATQNRRFQFTQNQKKEDSKTSTSVTSVNQASTSRLEGLQSENH
|(signal) |(mature peptide)
734 747

Score 5.0 at residue 44

Sequence LLLPLAPGAWGWA-RGAPRPPPSSPPLSIMGLMPLTKEVAKGSIGRGVLPAVELAIEQIRNESL
|(signal) |(mature peptide)
31 44

Score 4.9 at residue 497

FIG.3A

Sequence: ILSALTILGMIMA-SAFLFFNIKNRNQKLIKMSSPMNNLIILGGMLSYASIFLFGLDGSFVSE
|(signal) |(mature peptide)
484 497
Score 4.5 at residue 141

Sequence: LMVFGGVCPSVTS-IIAESLQGWNLVQLSFAATTPVLADKKKYPYFFRTVPSDNAVNPAILKLL
|(signal) |(mature peptide)
128 141
Score 4.4 at residue 734

Sequence: FCIVALVIIFCST-ITLCLVFVPKLITLRTNPDAATQNRRFQFTQNQKKEDSKTSTSVTSVNQA
|(signal) |(mature peptide)
721 734
Score 4.1 at residue 165

Sequence: VQLSFAATTPVLA-DKKKYPYFFRTVPSDNAVNPAILKLLKHYQWKRVGTLTQDVQRFSEVRND
|(signal) |(mature peptide)
152 165
Score 3.6 at residue 158

Sequence: SLQGWNLVQLSFA-ATTPVLADKKKYPYFFRTVPSDNAVNPAILKLLKHYQWKRVGTLTQDVQR
|(signal) |(mature peptide)
145 158

FIG.3B

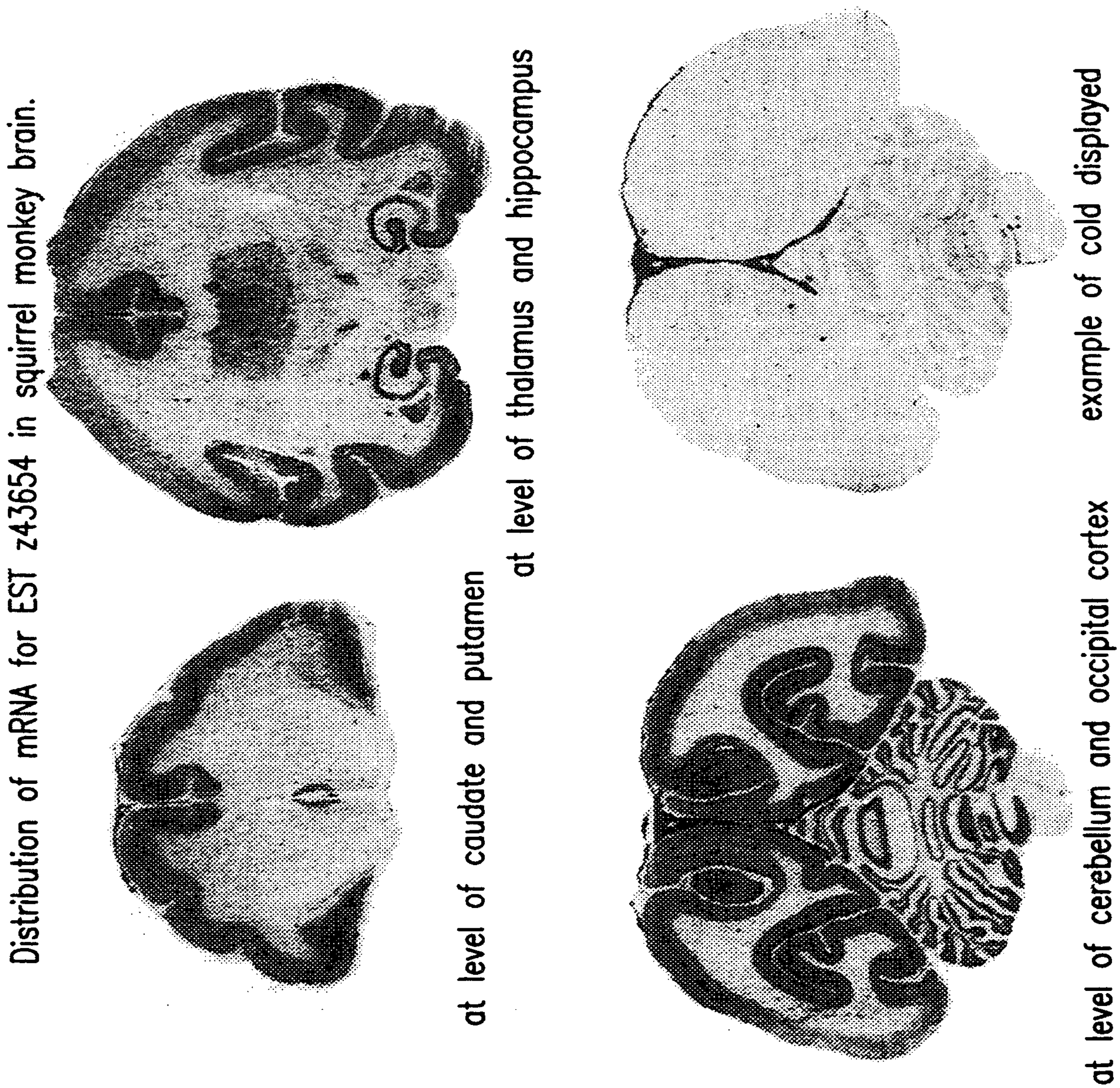
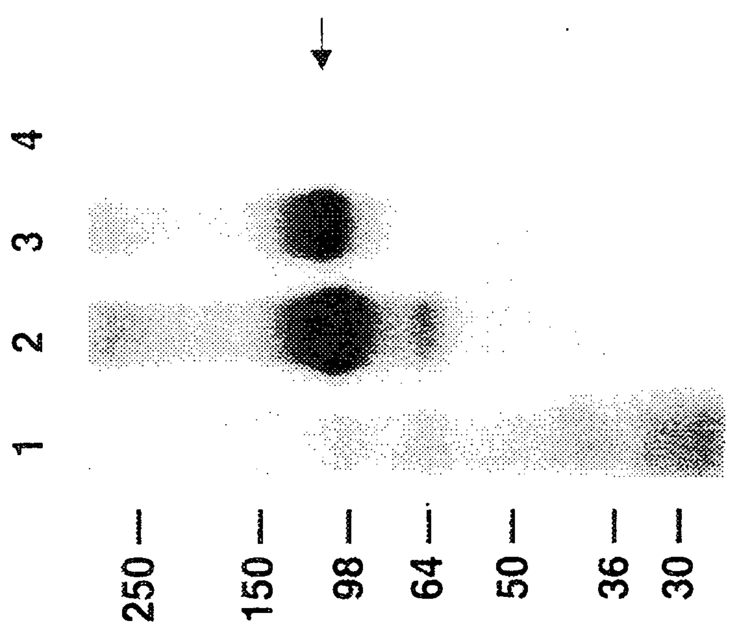
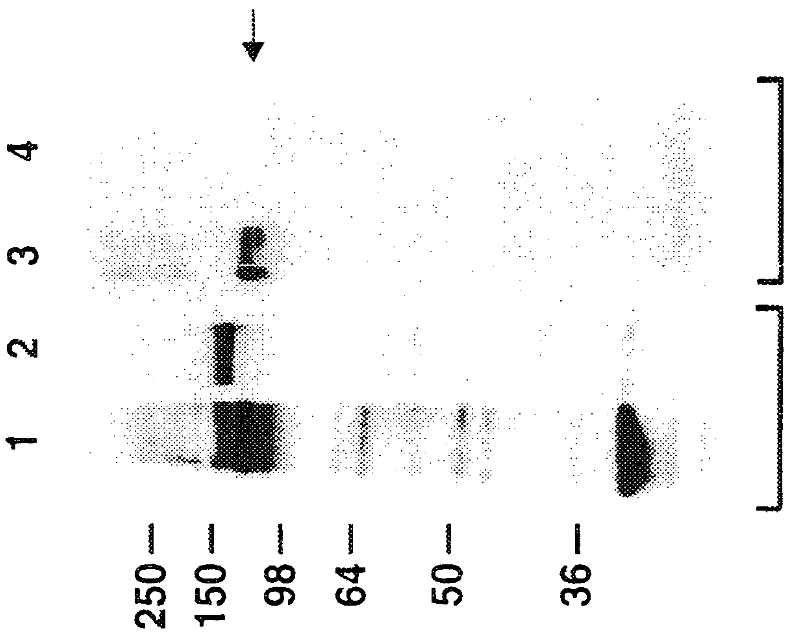

Distribution of mRNA for EST z43654 in squirrel monkey brain.
at level of caudate and putamen
at level of thalamus and hippocampus
at level of cerebellum and occipital cortex
example of cold displayed

FIG.4



1- HG20/PCR 3.1
2- FLAG-HG20/pcDNA 3:1
3- Mouse $GABA_B$/ pcDNA 3:1
4- pcDNA 3:1

FIG.5A



1- FLAG-HG20/pcDNA 3.1
2- pcDNA 3.1
3- FLAG-HG20/pcDNA 3.1
4- pcDNA 3.1

FIG.5B

```
PLTKEVAK-GSIGR-GVLPAVELAIEQIRNESLLRPYFLDLRLYDTECDNAKGLKAFYDA
:.:  ::. :.. : :.:     .X:::: . . .      :.  .::  :X   ... .   .
PVTGPVAQYGDMQRAGAL----MAIEQINKAGGVNGAQLEGVIYDDACDPKQAVAVANKV

IKYGPNHLMVFGGVCPSVTSIIAESLQGWNLVQLSFAATTPVLADKKKYPYFFRTVPSDN
.. :  .  .: : :: : :.  ..  .  ...... .::.: .. .. :  .:::.  ::
VNDGVK--FVVGHVCSSSTQPATDIYEDEGVLMITPSATAPEIT-SRGYKLIFRTIGLDN

AVNPAILKLL-KHYQWKRVGTLTQDVQRFSE-VRNDLTGVLYGEDIEISDTESFS---ND
  .:.  :.. ..:. : ...: .: :...: . ...   ..      :...  :...   .:
MQGPVAGKFIAERYKVKTIAVL-HDKQQYGEGIATEVKKTVEDAGIRVAVFEGLNAGDKD

PCTSVKKLKGNDVRII-LGQFDQNM
  . ..:::   :... .: .  .:
FNALISKLKKAGVQFVYFGGYHPEM
```

FIG.6

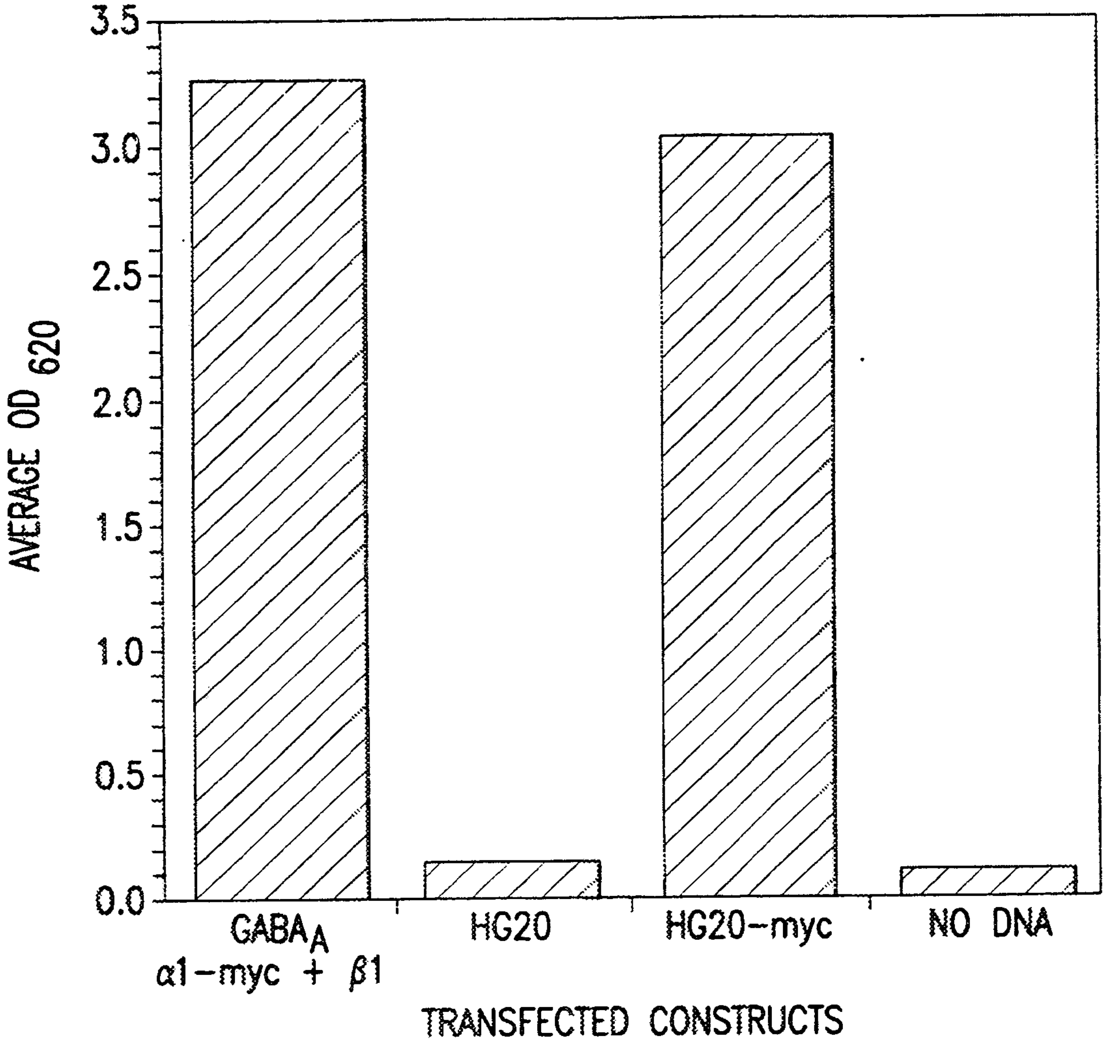

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
AVERAGE OD 620
GABAA
α1-myc + β1
HG20
HG20-myc
NO DNA
TRANSFECTED CONSTRUCTS

FIG.7

```
GABA-BR1b  MGPGGPCTPVGWPLPLLLVMAAGVAPVWASHSPHL---PRPHPRVPPHPSSERRAVYIGALFPMSGGWP-G--GQ  69
HG20       MASPRSSGQPGRPPPPPPPPARLILLLLLPLLLPLAPGAWGWARGAPRPPPSSPPLSIMGLMPLTKEVAKGSIGR  75
Consensus  M.........G.P.P.....A.............L........R..P.P........I..L.P.......G..G.  75

GABA-BR1b  ACQPAVEMALEDVNSRRDILPDYELKLIHHDSKCDPGQATKYLYELLYNDPIKIILMPG-CSSVSTLVAEAARMW  143
HG20       GVLPAVELAIEQIRN-ESLLRPYFLDLRLYDTECDNAKGLKAFYDAIKYGPNHLMVFGGVCPSVTSIIAESLQGW  149
Consensus  ...PAVE.A.E........L..Y.L.L...D..CD.....K..Y......P.......G.C.SV....AE....W  150

GABA-BR1b  NLIVLSYGSSSPALSNRQRFPTFFRTHPSATLHNPTRVKLFEKWGWKKIATIQQTTEVFTSTLDDLEERVKEAGI  218
HG20       NLVQLSFAATTPVLADKKKYPYFFRTVPSDNAVNPAILKLLKHYQWKRVGTLTQDVQRFSEVRNDLTGVLYGEDI  224
Consensus  NL..LS.....P.L......P.FFRT.PS....NP...KL.....WK...T..Q....F.....DL........I  225

GABA-BR1b  EITFRQSFFSDPAVPVKNLKRQDARIIVGLFYETEARKVFCEVYKERLFGKKYVWFLIGWYADNWF---KTYDPS  290
HG20       EISDTESFSNDPCTSVKKLKGNDVRIILGQFDQNMAAKVFCCAYEENMYGSKYQWIIPGWYEPSWWEQVHTEANS  299
Consensus  EI....SF..DP...VK.LK...D.RII.G.F...A.KVFC..Y.E...G.KY.W...GWY...W.....T...S  300

GABA-BR1b  INCTVEEMTEAVEGHITTEIVMLNPANTRSISNMTSQEFVEKLTKRLKRHPEETGGFQEAPLAYDAIWALALALN  365
HG20       SRCLRKNLLAAMEGYIGVDFEPLSSKQIKTISGKTPQQY-EREYNN-KRSGVGPSKFHGYAYDGIWVIAKTLQRA  372
Consensus  ..C.......A.EG.I......L.......IS..T.Q...E......KR.......F...........A..L...  375

GABA-3R1b  KTSGGGGRSGVRLEDFNYNNQTITDQIYRAMNSSSFEGVSGHVVFDASGSRMAWTLIEQLQGGSYKKIGYYDSTK  440
HG20       METLHASSRHQRIQDFNYTDHTLGRIILNAMNETNFFGVTGQVVFR-NGERMGTIKFTQFQDSREVKVGEYNAVA  446
Consensus  ...........R..DFNY...T...I....AMN..F.GV.G.VVF...G.RM......Q.Q.....K.G.Y....  450

GABA-BR1b  DDLSWSKTDKWIGGSPPADQTLVIKTFRFLSQKLFISVSVLSSLGIVLAVVC---LSFNIYNSHVRYIQNSQPNL  512
HG20       DTLEIINDTIRFQGSEPPKDKTII--LEQLRKISLPLYSILSALTILGMIMASAFLFFNIKNRNQKLIKMSSPYM  519
Consensus  D.L..........GS.P......I.....L........S.LS.L.I.........L.FNI.N.....I..S.P..  525
```

FIG.8A

```
GABA-BR1b NNLTAVGCSLALAAVFPLGLDGYHIGRSQFPFVCQARLWLLGLGFSLGYGSMFTKIWWVHTVFTKKEEKKEWRKT 587
HG20      NNLIILGGMLSYASIFLFGLDGSFVSEKTFETLCTVRTWILTVGYTTAFGAMFAKTWRVHAIF--KNVKMK-KKI 591
Consensus NNL...G..L..A..F..GLDG......F...C..R.W.L..G.....G.MF.K.W.VH..F..K..K....K. 600

GABA-BR1b LEPWKLYATVGLLVGMDVLTLAIWQIVDPLHRTIETFAKEEPKEDIDVSILPQLEHCSSKKMNTWLGIFYGYKGL 662
HG20      IKDQKLLVIVGGMLLIDLCILICWQAVDPLRRTVEKYSMEPDPAGRDISIRPLLEHCENTHMTIWLGIVYAYKGL 666
Consensus ....KL...VG.....D...L..WQ.VDPL.RT.E....E......D.SI.P.LEHC....M..WLGI.Y.YKGL 675

GABA-BR1b LLLLGIFLAYETKSVSTEKINDHRAVGMAIYNVAVLCLITAPVTMILSSQQDAAFAFASLAIVFSSYITLVVLFV 737
HG20      LMLFGCFLAWETRNVSIPALNDSKYIGMSVYNVGIMCIIGAAVSFLTRDQPNVQFCIVALVIIFCSTITLCLVFV 741
Consensus L.L.G.FLA.ET..VS....ND....GM..YNV...C.I.A.V......Q....F....L.I.F.S.ITL...FV 750

GABA-BR1b PKMRRLITRGE------W----QSETQDTMKTGSS-TNNNEEEKSRL--LEKE----------NRELEKI---- 784
HG20      PKLITLRTNPDAATQNRRFQFTQNQKKEDSKTSTSVTSVNQASTSRLEGLQSENHRLRMKITELDKDLEEVTMQL 816
Consensus PK...L.T..............Q.......KT..S.T..N....SRL..L..E .............LE...... 825

GABA-BR1b --IAEKE--------ERVSE----------------LRHQLQSRQQLRSRRHPPTPP--DPSGG---------- 820
HG20      QDTPEKTTYIKQNHYQELNDILNLGNFTESTDGGKAILKNHLDQNPQLQWNTTEPSRTCKDPIEDINSPEHIQRR 891
Consensus ....EK...........................L...L....QL......P.....DP............. 900

GABA-BR1b ------------LPR-GPSEPPDRLSC----DGSRVHLL---YK------ 844
HG20      LSLQLPILHHAYLPSIGGVDASCVSPCVSPTASPRHRHVPPSFRVMVSGL 941
Consensus ............LP..G.........C.......R............... 950
```

FIG.8B

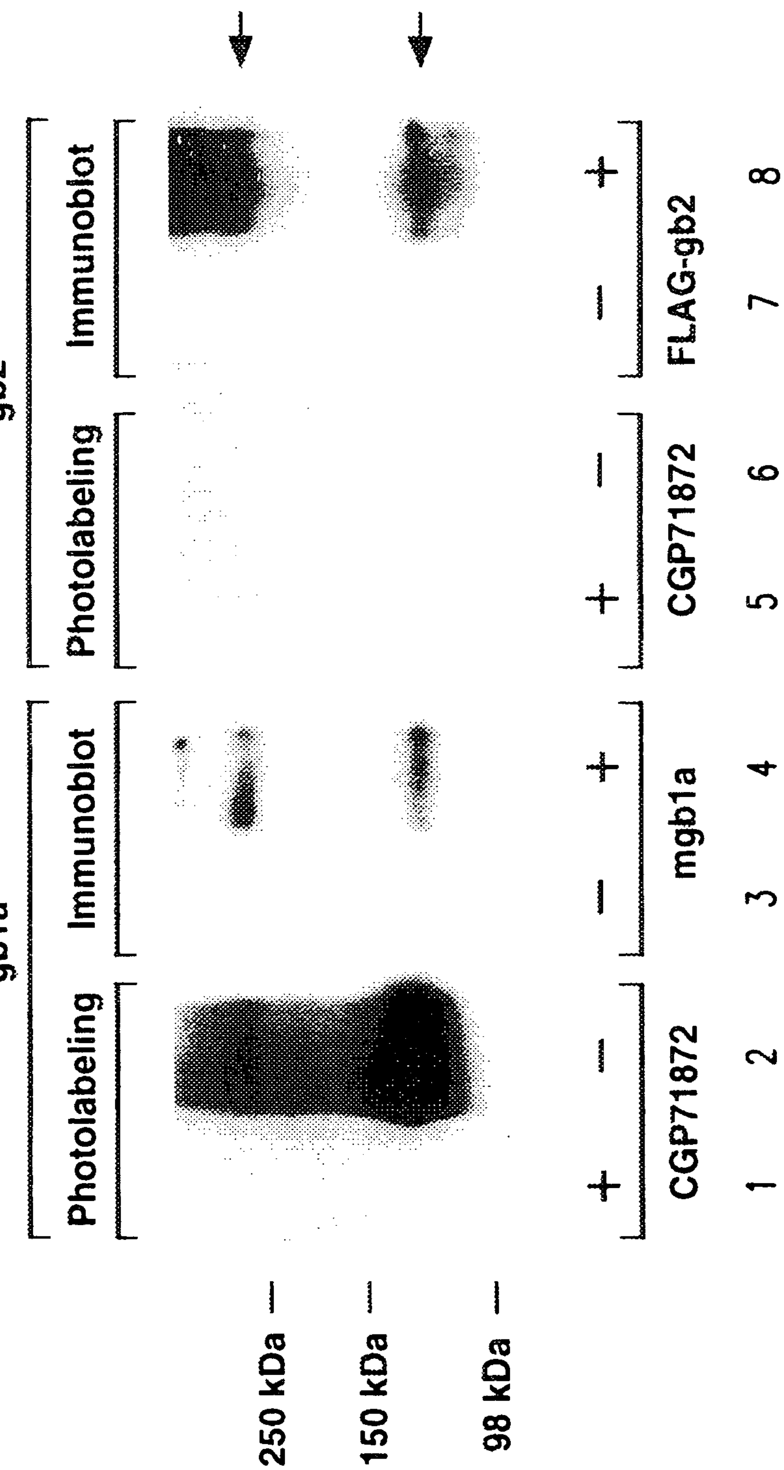

gb1a
gb2
Photolabeling
Immunoblot
Photolabeling
Immunoblot
250 kDa
150 kDa
98 kDa
+
−
−
+
+
−
−
+
CGP71872
mgb1a
CGP71872
FLAG-gb2
1
2
3
4
5
6
7
8

FIG.9

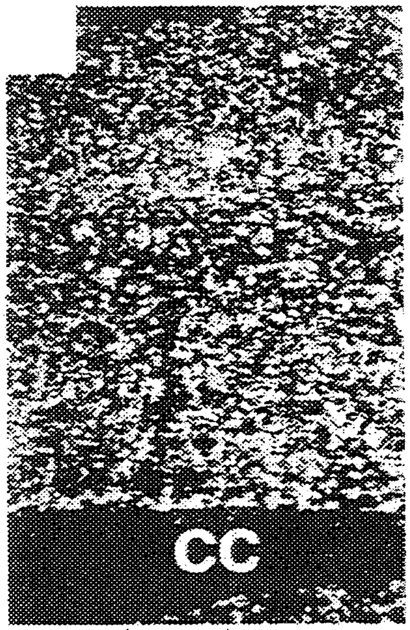


FIG.10A

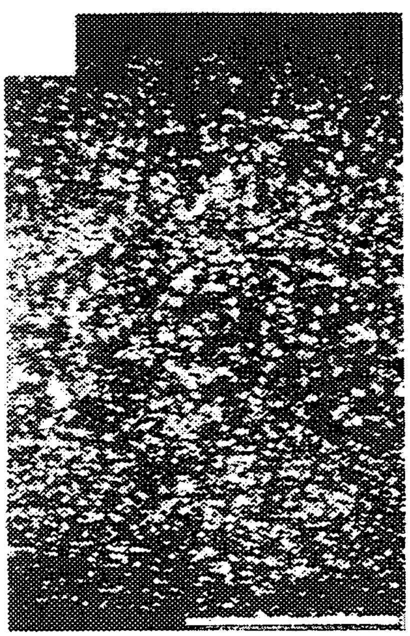


FIG.10B

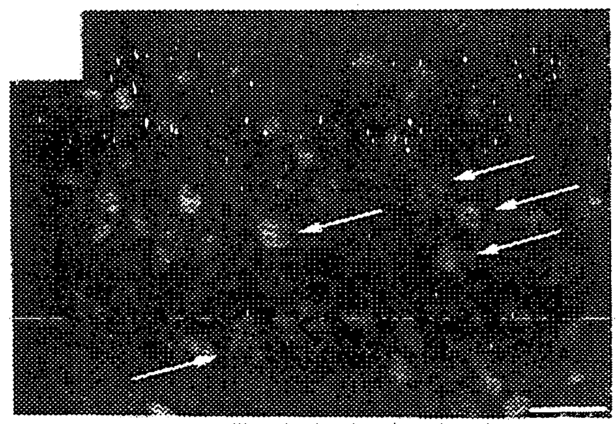


FIG.10C

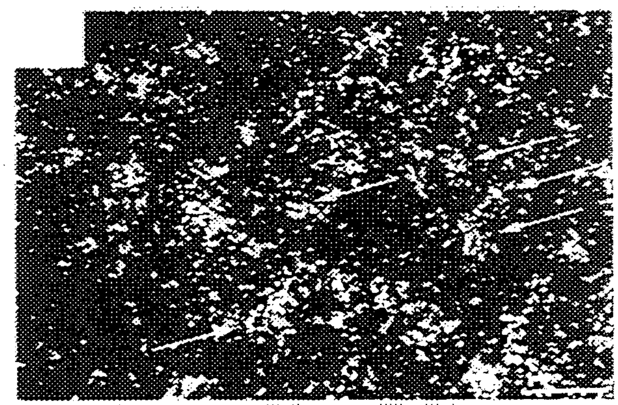


FIG.10D

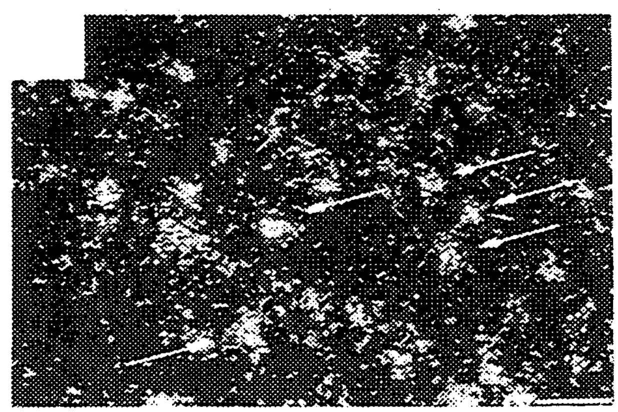


FIG.10E

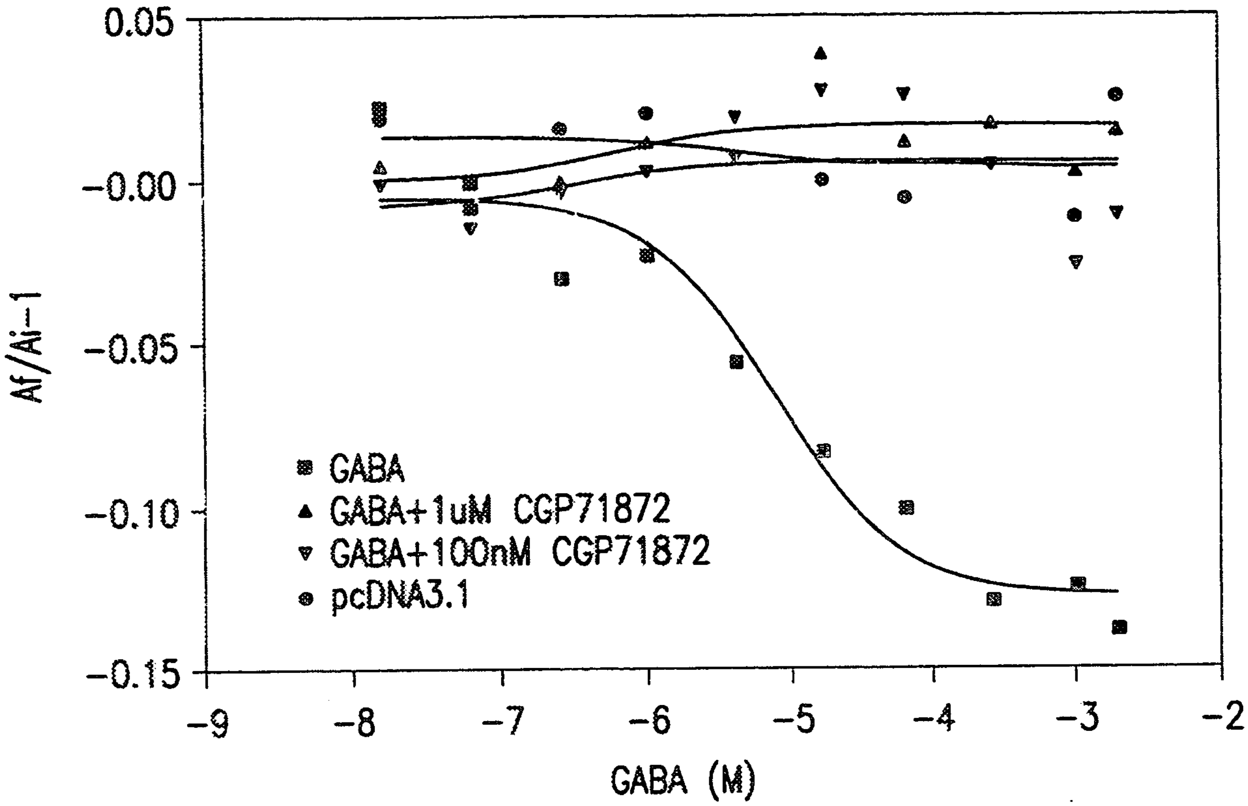


FIG. 11A

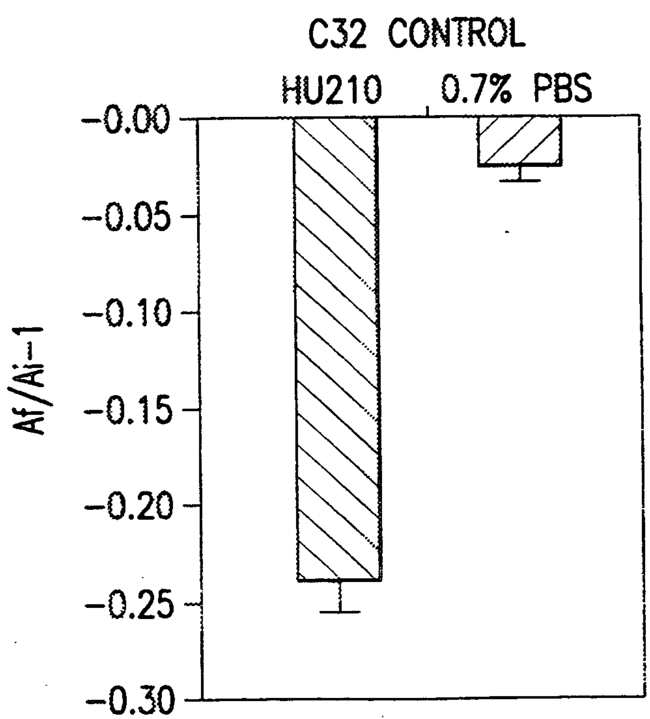


FIG. 11B

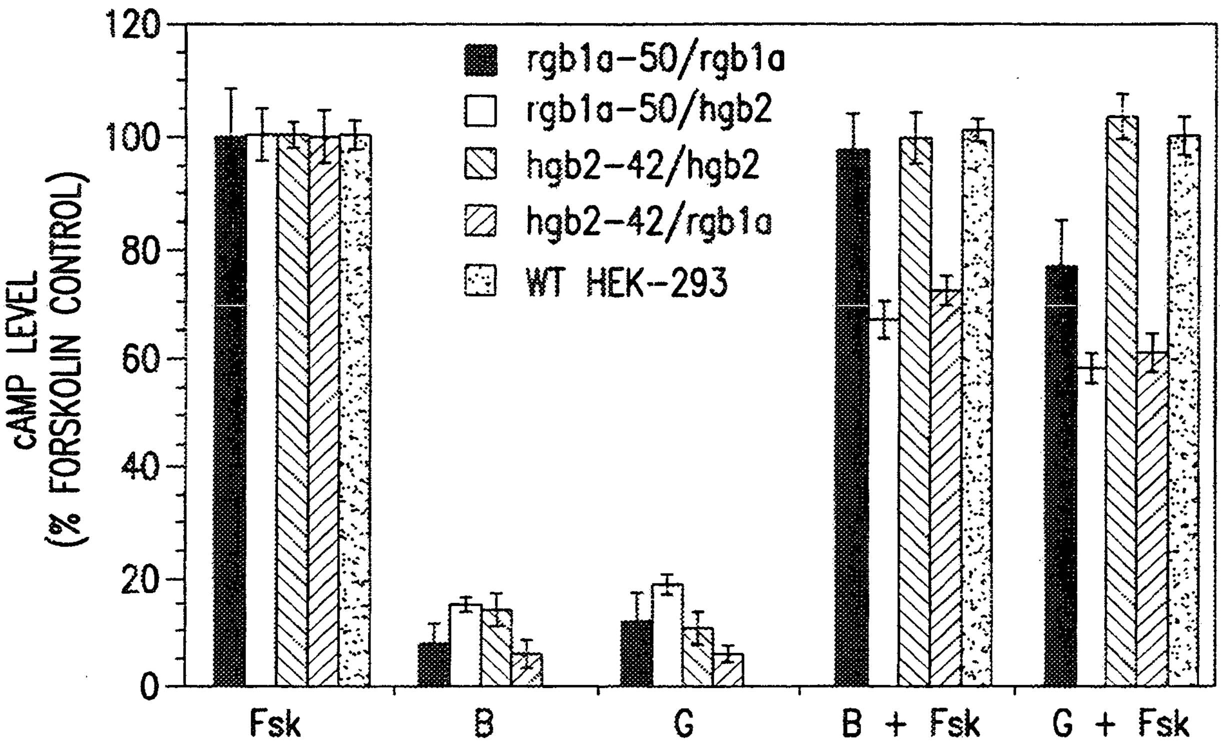

120
100
80
60
40
20
0
cAMP LEVEL
(% FORSKOLIN CONTROL)
rgb1a-50/rgb1a
rgb1a-50/hgb2
hgb2-42/hgb2
hgb2-42/rgb1a
WT HEK-293
Fsk
B
G
B + Fsk
G + Fsk

FIG. 12

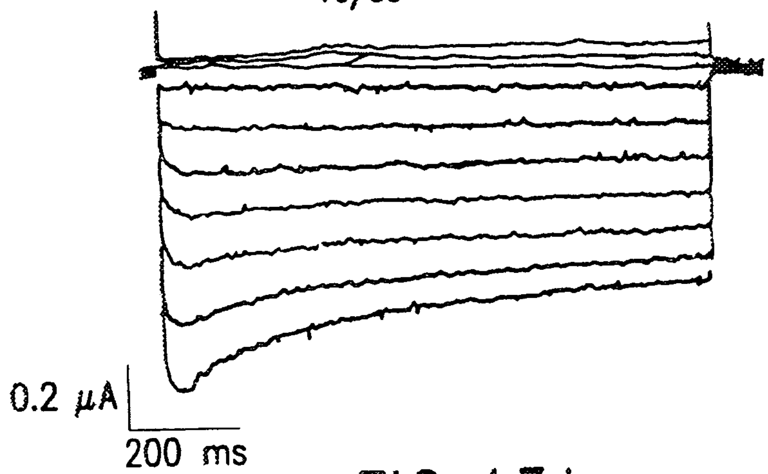


FIG. 13A

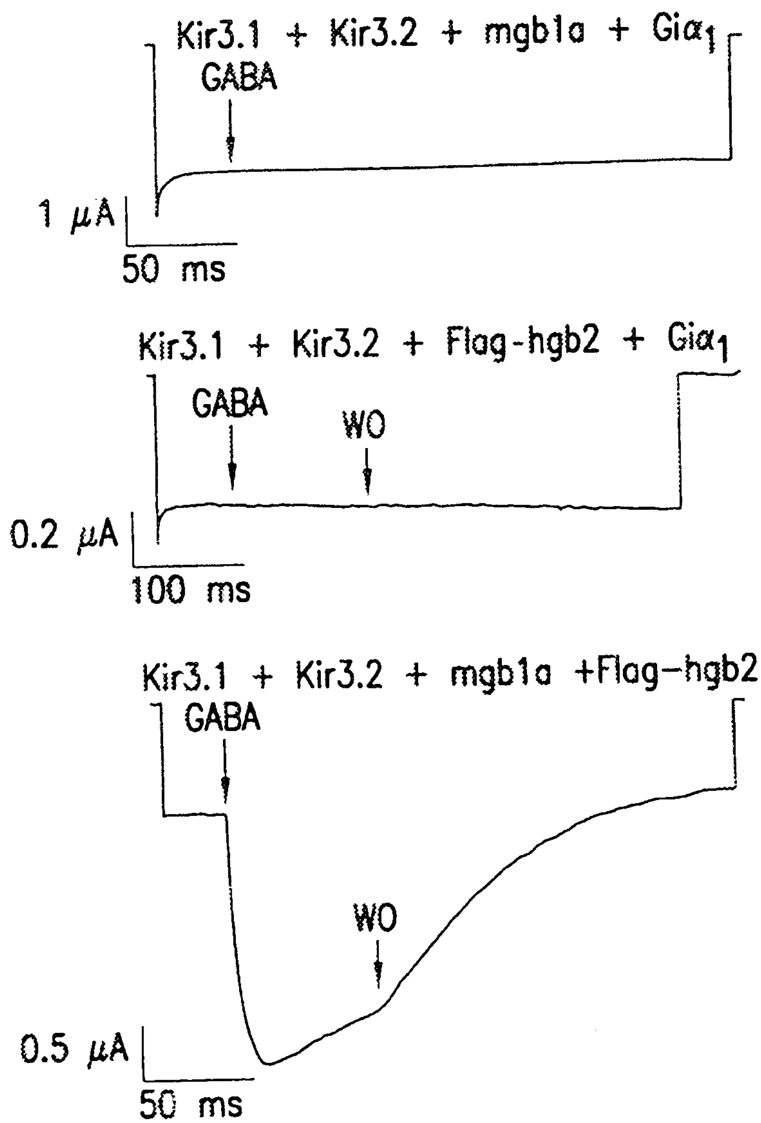


FIG. 13B

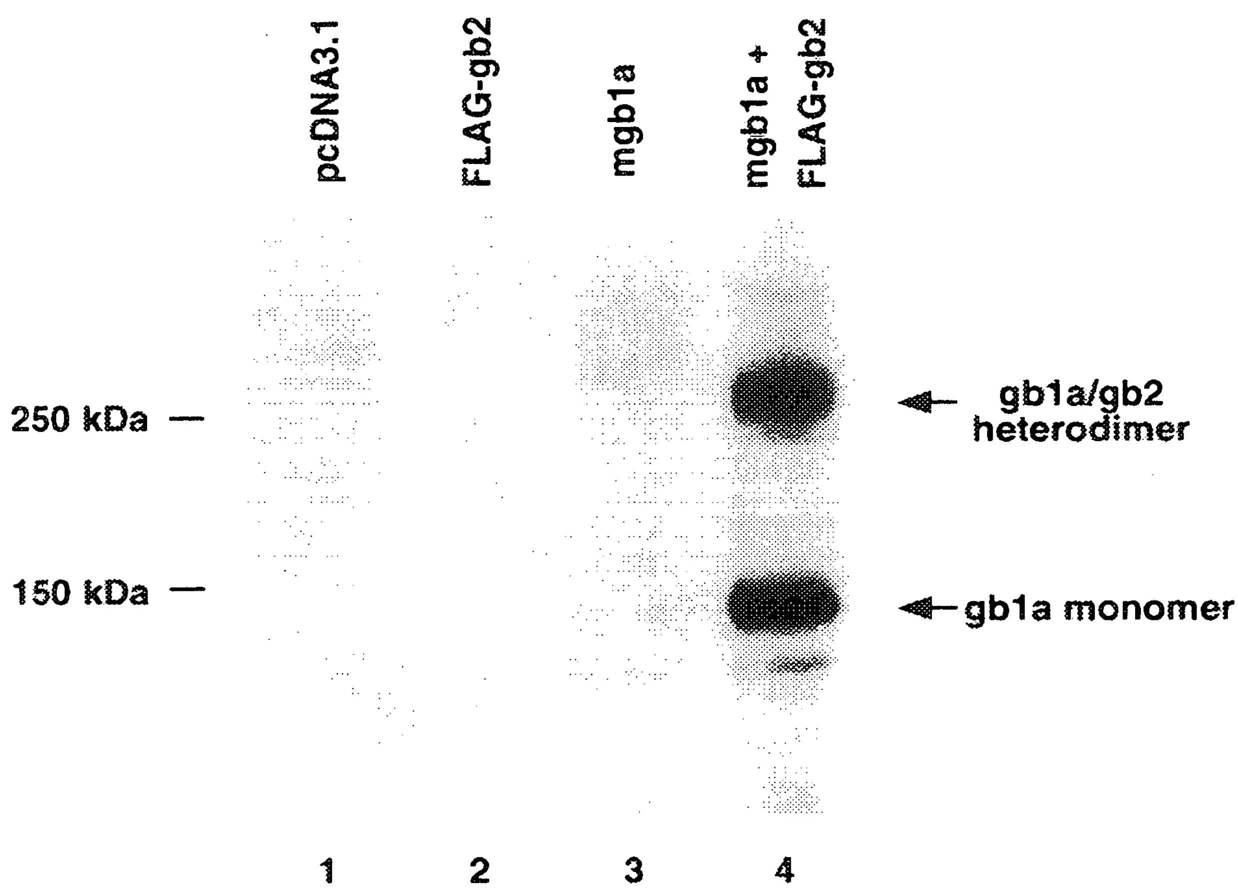

pcDNA3.1
FLAG-gb2
mgb1a
mgb1a + FLAG-gb2
250 kDa
150 kDa
gb1a/gb2 heterodimer
gb1a monomer
1
2
3
4

FIG. 14

1 atgctgctgc tgctgcttct gcttctcttc ctccgccccc tgggcgctgg cggggctcag
61 accccccaacg tcacctcgga aggttgccag attatacatc cgccctggga aggtggcatc
121 aggtaccgtg gcttgattcg cgaccaggtg aaggccatca atttcctgcc tgtggactat
181 gagattgaat atgtgtgccg gggcgaacgc gaggtggtgg ggcccaaggt gcgcaagtgc
241 ctggccaacg gctcctggac ggatatggac acacccagtc gctgtgtccg aatctgctcc
301 aagtcttatt tgaccctgga aaatgggaag gttttcctga cgggtgggga cctcccagct
361 ctggatggag cccgggtgga tttccgatgt gaccctgact tccatctggt gggcagctcc
421 cggagcatct gtagtcaggg ccagtggagc accccccaagc ccccactgcca ggtgaatcga
481 acgccacact cagaacggcg tgcagtatac atcggggcgc tgtttcccat gagcgggggc
541 tggccggggg gccaggcctg ccagcctgcg gtggagatgg cgctggagga cgttaacagc
601 cgcagagaca tcctgccgga ctacgagctc aagcttatcc accacgacag caagtgcgac
661 ccagggcaag ccaccaagta cttgtatgaa ctactctaca acgaccccat caagatcatc
721 ctcatgcccg gctgcagctc tgtgtccaca ctggtagccg aggctgcccg gatgtggaac
781 cttattgtgc tctcatatgg ctccagctca ccagccttgt caaaccgaca gcggtttcca
841 acgttctttc ggacacatcc atccgccaca ctccacaatc ccacccgggt gaaactcttc
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961 acactggatg acctggagga gcgagtgaaa gaggctggga ttgagatcac ttttcgacag
1021 agtttcttct cagatccagc tgtgcctgtt aaaaacctga agcgtcaaga tgctcgaatc
1081 atcgtgggac ttttctatga gaccgaagcc cggaaagttt tttgtgaggt ctataaggaa
1141 cggctctttg ggaagaagta tgtctggttt ctcatcgggt ggtatgctga caactggttc
1201 aaaacctatg acccgtcaat caattgtaca gtagaagaga tgactgaggc ggtggagggc
1261 catatcacca cggagattgt catgctgaac cctgccaaca cccgaagcat ttccaacatg
1321 acatcacagg aatttgtgga gaaactaacc aagcggctga aaagacaccc tgaggagact
1381 ggaggcttcc aggaggcacc actggcctat gatgctattt gggccttggc tttggccttg
1441 aacaagacct ctggaggagg tggccgttca ggagtgcgcc tggaggactt taactacaac
1501 aaccagacca ttacagacca aatctaccgg gccatgaact cctcctcctt tgagggtgtt
1561 tctggccacg tggtctttga tgccagcggc tcccggatgg catggacgct tatcgagcag
1621 ctacagggcg gcagctacaa gaagatcggc tactacgaca gcaccaagga tgatctttcc
1681 tggtccaaaa cagacaagtg gatcggaggg tctcccccag ccgaccagac cttggtcatc
1741 aagacattcc gtttcctgtc acagaaactc tttatctccg tctcagttct ctccagcctg
1801 ggcattgttc ttgctgttgt ctgtctgtcc tttaacatct acaactccca cgctcgttat
1861 atccagaatt cccagcccaa cctgaacaat ctgactgctg tgggctgctc actggcactg
1921 gctgttgtct tccctctcgg gctggatggt taccacatag ggagaagcca gttcccgttt
1981 gtctgccagg cccgcctttg gctcttgggc ttgggcttta gtctgggcta tggctctatg
2041 ttcaccaaga tctggtgggt ccacacagtc ttcacgaaga aggaggagaa gaaggagtgg
2101 aggaagaccc tagagccctg gaaactctat gccactgtgg gcctgctggt gggcatggat
2161 gtcctgactc ttgccatctg gcagattgtg gaccccttgc accgaaccat tgagactttt
2221 gccaaggagg aaccaaagga agacatcgat gtctccattc tgccccagtt ggagcactgc
2281 agctccaaga agatgaatac gtggcttggc attttctatg gttacaaggg gctgctgctg
2341 ctgctgggaa tctttcttgc ttacgaaacc aagagcgtgt ccactgaaaa gatcaatgac
2401 cacagggccg tgggcatggc tatctacaat gtcgcggtcc tgtgtctcat cactgctcct
2461 gtgaccatga tcctttccag tcagcaggac gcagcctttg cctttgcctc tctggccatc
2521 gtgttctctt cctacatcac tctggttgtg ctctttgtgc ccaagatgcg caggctgatc
2581 acccgagggg aatggcagtc tgaaacgcag gacaccatga aaacaggatc atccaccaac
2641 aacaacgagg aagagaagtc ccgactgttg gagaaggaaa accgagaact ggaaaagatc
2701 atcgctgaga aagaggagcg cgtctctgaa ctgcgccatc agctccagtc tcggcagcaa
2761 ctccgctcac ggcgccaccc cccaacaccc ccagatccct ctgggggcct tcccagggga
2821 ccctctgagc cccctgaccg gcttagctgt gatgggagtc gagtacattt gctttacaag
2881 tga

FIG.15

MLLLLLLLLFLRPLGAGGAQTPNVTSEGCQIIHPPWEGGIRYRGLIRDQVKAINFLPVDY
EIEYVCRGEREVVGPKVRKCLANGSWTDMDTPSRCVRICSKSYLTLENGKVFLTGGDLPA
LDGARVDFRCDPDFHLVGSSRSICSQGQWSTPKPHCQVNRTPHSERRAVYIGALFPMSGG
WPGGQACQPAVEMALEDVNSRRDILPDYELKLIHHDSKCDPGQATKYLYELLYNDPIKII
LMPGCSSVSTLVAEAARMWNLIVLSYGSSSPALSNRQRFPTFFRTHPSATLHNPTRVKLF
EKWGWKKIATIQQTTEVFTSTLDDLEERVKEAGIEITFRQSFFSDPAVPVKNLKRQDARI
IVGLFYETEARKVFCEVYKERLFGKKYVWFLIGWYADNWFKTYDPSINCTVEEMTEAVEG
HITTEIVMLNPANTRSISNMTSQEFVEKLTKRLKRHPEETGGFQEAPLAYDAIWALALAL
NKTSGGGGRSGVRLEDFNYNNQTITDQIYRAMNSSSFEGVSGHVVFDASGSRMAWTLIEQ
LQGGSYKKIGYYDSTKDDLSWSKTDKWIGGSPPADQTLVIKTFRFLSQKLFISVSVLSSL
GIVLAVVCLSFNIYNSHARYIQNSQPNLNNLTAVGCSLALAVVFPLGLDGYHIGRSQFPF
VCQARLWLLGLGFSLGYGSMFTKIWWVHTVFTKKEEKKEWRKTLEPWKLYATVGLLVGMD
VLTLAIWQIVDPLHRTIETFAKEEPKEDIDVSILPQLEHCSSKKMNTWLGIFYGYKGLLL
LLGIFLAYETKSVSTEKINDHRAVGMAIYNVAVLCLITAPVTMILSSQQDAAFAFASLAI
VFSSYITLVVLFVPKMRRLITRGEWQSETQDTMKTGSSTNNNEEEKSRLLEKENRELEKI
IAEKEERVSELRHQLQSRQQLRSRRHPPTPPDPSGGLPRGPSEPPDRLSCDGSRVHLLYK

FIG.16

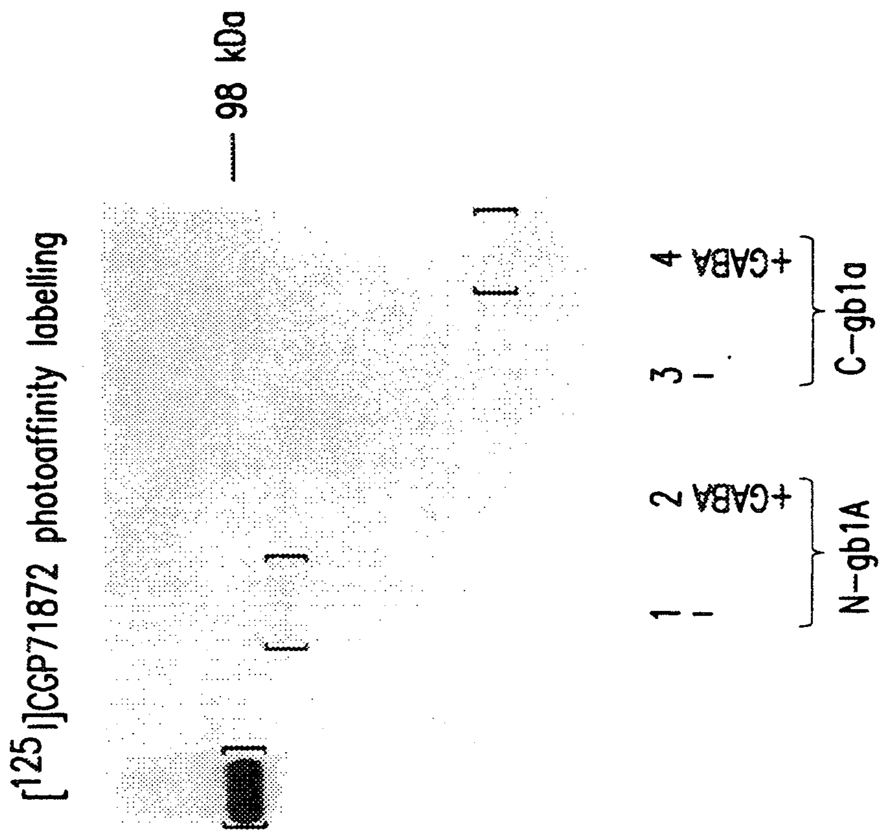

FIG.17B

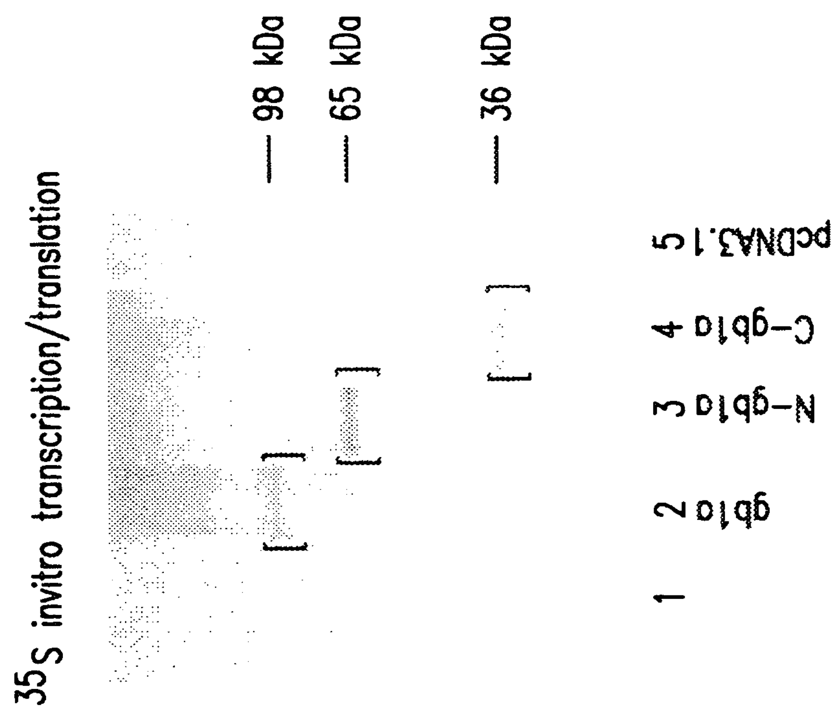

FIG.17A

MLLLLLLAPLFLRPPGAGGAHTPNATSEGCQIIHPPWEGGIRYRGLTRDQV
KAINFLPVDYEIEYVCRGEREVVGPKVRKCLANGSWTDMDTPSRCVRICS
KSYLTLENGKVFLTGGDLPALDGARADFRCDPDFHLVGSSRSICSQGQWST
PKPHCQVNRTPHSERRAVYIGALFPMSGGWPGGQACQPAVEMALEDVNS
RRDILPDYELKLIHHDSKCDPGQATKYLYELLYNDPIKIILMPGCSSVSTLV
AEAARMWNLIVLSYGSSSPALSNRORFPTFFRTHPSATLHNPTRVKLFEKW
GWKKIATIQQTTEVFTSTLDDLEERVKEAGIEITFRQSFFSDPAVPVKNLKRQ
DARIIVGLFYETEARKVFCEVYKERLFGKKYVWFLIGWYADNWFKIYDPS
INCTVDEMTEAVEGHITTEIVMLNPANTRSISNMTSQEFVEKLTKRLKRHPE
ETGGFQEAPLAYDAIWALALALNKTSGGGGRSGVRLEDFNYNNQTITDQI
YRAMNSSSFEGVSGHVVFDASGSRMAWTLIEQLQGGSYKKIGYYDSTKDD
LSWSKTDKWIGGSPPADQTLVIKTFRFLSQKLFISVSVLSSLGIVLAVVCLSF
NIYNSHVRYIQNSQPNLNNLTAVGCSLALAAVFPLGLDGYHIGRNQFPFV
CQARLWLLGLGFSLGYGSMFTKIWWVHTVFTKKEEKKEWRKTLEPWKLY
ATVGLLVGMDVLTLAIWQIVDPLHRTIETFAKEEPKEDIDVSILPQLEHCSS
RKMNTWLGIFYGYKGLLLLLGIFLAYETKSVSTEKINDHRAVGMAIYNVA
VLCLITAPVTMILSSQQDAAFAFASLAIVFSSYITLVVLFVPKMRRLITRGE
WQSEAQDTMKTGSSTNNNEEEKSRLLEKENRELEKIIAEKEERVSELRHQLQ
SRQQLRSRRHPPTPPEPSGGLPRGPPEPPDRLSCDGSRVHLLYK

FIG.18A

1 atgttgctgc tgctgctact ggcgccactc ttcctccgcc ccccgggcgc gggcggggcg
61 catacccca acgccacctc agaaggttgc cagatcatac acccgccctg ggaaggggc
121 atcaggtacc ggggcctgac tcgggaccag gtgaaggcta tcaacttcct gccagtggac
181 tatgagattg agtatgtgtg ccggggggag cgcgaggtgg tggggcccaa ggtccgcaag
241 tgcctggcca acggctcctg gacagatatg gacacaccca gccgctgtgt ccgaatctgc
301 tccaagtctt atttgaccct ggaaaatggg aaggttttcc tgacgggtgg ggacctccca
361 gctctggacg gagcccggc ggatttccgg tgtgaccccg acttccatct ggtgggcagc
421 tcccggagca tctgtagtca gggccagtgg agcaccccca agccccactg ccaggtgaat
481 cgaacgccac actcagaacg gcgcgcagtg tacatcgggg cactgtttcc catgagcggg
541 ggctggccag gggccaggc ctgccagccc gcggtggaga tggcgctgga ggacgtgaat
601 agccgcaggg acatcctgcc ggactatgag ctcaagctca tccaccacga cagcaagtgt
661 gatccaggcc aagccaccaa gtacctatat gagctgctct acaacgaccc tatcaagatc
721 atccttatgc ctggctgcag ctctgtctcc acgctggtgg ctgaggctgc taggatgtgg
781 aacctcattg tgctttccta tggctccagc tcaccagccc tgtcaaaccg gcagcgtttc
841 cccactttct tccgaacgca cccatcagcc acactccaca accctacccg cgtgaaactc
901 tttgaaaagt ggggctggaa gaagattgct accatccagc agaccactga ggtcttcact
961 tcgactctgg acgacctgga ggaacgagtg aaggaggctg gaattgagat tactttccgc
1021 cagagtttct tctcagatcc agctgtgccc gtcaaaaacc tgaagcgcca ggatgcccga
1081 atcatcgtgg gacttttcta tgagactgaa gcccggaaag ttttttgtga ggtgtacaag
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1321 atgacatccc aggaatttgt ggagaaacta accaagcgac tgaaaagaca ccctgaggag
1381 acaggaggct tccaggaggc accgctggcc tatgatgcca tctgggcctt ggcactggcc
1441 ctgaacaaga catctggagg aggcggccgt tctggtgtgc gcctggagga cttcaactac
1501 aacaaccaga ccattaccga ccaaatctac cgggcaatga actcttcgtc ctttgagggt
1561 gtctctggcc atgtggtgtt tgatgccagc ggctctcgga tggcatggac gcttatcgag
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1681 tcctggtcca aaacagataa atggattgga gggtcccccc cagctgacca gaccctggtc
1741 atcaagacat tccgcttcct gtcacagaaa ctctttatct ccgtctcagt tctctccagc
1801 ctgggcattg tcctagctgt tgtctgtctg tcctttaaca tctacaactc acatgtccgt
1861 tatatccaga actcacagcc caacctgaac aacctgactg ctgtgggctg ctcactggct
1921 ttagctgctg tcttccccct ggggctcgat ggttaccaca ttgggaggaa ccagtttcct
1981 ttcgtctgcc aggcccgcct ctggctcctg ggcctgggct ttagtctggg ctacggttcc
2041 atgttcacca agatttggtg ggtccacacg gtcttcacaa agaaggaaga aaagaaggag
2101 tggaggaaga ctctggaacc ctggaagctg tatgccacag tgggcctgct ggtgggcatg
2161 gatgtcctca ctctcgccat ctggcagatc gtggaccctc tgcaccggac cattgagaca
2221 tttgccaagg aggaacctaa ggaagatatt gacgtctcta ttctgcccca gctggagcat
2281 tgcagctcca ggaagatgaa tacatggctt ggcattttct atggttacaa ggggctgctg
2341 ctgctgctgg gaatcttcct tgcttatgag accaagagtg tgtccactga gaagatcaat
2401 gatcaccggg ctgtgggcat ggctatctac aatgtggcag tcctgtgcct catcactgct
2461 cctgtcacca tgattctgtc cagccagcag gatgcagcct ttgcctttgc ctctcttgcc
2521 atagttttct cctcctatat cactcttgtt gtgctctttg tgcccaagat gcgcaggctg
2581 atcacccgag gggaatggca gtcggaggcg caggacacca tgaagacagg gtcatcgacc
2641 aacaacaacg aggaggagaa gtcccggctg ttggagaagg agaaccgtga actggaaaag
2701 atcattgctg agaaagagga gcgtgtctct gaactgcgcc atcaactcca gtctcggcag
2761 cagctccgct cccggcgcca cccaccgaca cccccagaac cctctggggg cctgcccagg
2821 ggacccctg agccccccga ccggcttagc tgtgatggga gtcgagtgca tttgctttat
2881 aagtga

FIG. 18B

1 atgctgctgc tgctgctggc gccactcttc ctccgccccc cgggcgcggg cggggcgcag
61 acccccaacg ccacctcaga aggttgccag atcatacacc cgccctggga agggggcatc
121 aggtaccggg gcctgactcg ggaccaggtg aaggctatca acttcctgcc agtggactat
181 gagattgagt atgtgtgccg gggggagcgc gaggtggtgg ggcccaaggt ccgcaagtgc
241 ctggccaacg gctcctggac agatatggac acacccagcc gctgtgtccg aatctgctcc
301 aagtcttatt tgaccctgga aaatgggaag gttttcctga cgggtgggga cctcccagct
361 ctggacggag cccgggtgga tttccggtgt gaccccgact tccatctggt gggcagctcc
421 cggagcatct gtagtcaggg ccagtggagc acccccaagc ccccactgcca ggtgaatcga
481 acgccacact cagaacggcg cgcagtgtac atcggggcac tgtttcccat gagcgggggc
541 tggccagggg gccaggcctg ccagcccgcg gtggagatgg cgctggagga cgtgaatagc
601 cgcagggaca tcctgccgga ctatgagctc aagctcatcc accacgacag caagtgtgat
661 ccaggccaag ccaccaagta cctatatgag ctgctctaca acgaccctat caagatcatc
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781 ctcattgtgc tttcctatgg ctccagctca ccagccctgt caaaccggca gcgtttcccc
841 actttcttcc gaacgcaccc atcagccaca ctccacaacc ctacccgcgt gaaactcttt
901 gaaaagtggg gctggaagaa gattgctacc atccagcaga ccactgaggt cttcacttcg
961 actctggacg acctggagga acgagtgaag gaggctggaa ttgagattac tttccgccag
1021 agtttcttct cagatccagc tgtgcccgtc aaaaacctga agcgccagga tgcccgaatc
1081 atcgtgggac ttttctatga gactgaagcc cggaaagttt tttgtgaggt gtacaaggag
1141 cgtctctttg ggaagaagta cgtctggttc ctcattgggt ggtatgctga caattggttc
1201 aagatctacg acccttctat caactgcaca gtggatgaga tgactgaggc ggtggagggc
1261 cacatcacaa ctgagattgt catgctgaat cctgccaata cccgcagcat ttccaacatg
1321 acatcccagg aatttgtgga gaaactaacc aagcgactga aaagacaccc tgaggagaca
1381 ggaggcttcc aggaggcacc gctggcctat gatgccatct gggccttggc actggccctg
1441 aacaagacat ctggaggagg cggccgttct ggtgtgcgcc tggaggactt caactacaac
1501 aaccagacca ttaccgacca aatctaccgg gcaatgaact cttcgtcctt tgagggtgtc
1561 tctggccatg tggtgtttga tgccagcggc tctcggatgg catggacgct tatcgagcag
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1681 tggtccaaaa cagataaatg gattggaggg tccccccag ctgaccagac cctggtcatc
1741 aagacattcc gcttcctgtc acagaaactc tttatctccg tctcagttct ctccagcctg
1801 ggcattgtcc tagctgttgt ctgtctgtcc tttaacatct acaactcaca tgtccgttat
1861 atccagaact cacagcccaa cctgaacaac ctgactgctg tgggctgctc actggcttta
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2641 aacaacgagg aggagaagtc ccggctgttg gagaaggaga accgtgaact ggaaaagatc
2701 attgctgaga aagaggagcg tgtctctgaa ctgcgccatc aactccagtc tcggcagcag
2761 ctccgctccc ggcgccaccc accgacaccc ccagaaccct ctggggggcct gcccaggggga
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2881 tgagggtagg gtgagggagg acaggccagt agggggaggg aaagggagag gggaagggca
2941 ggggactcag gaagcagggg gtccccatcc ccagctggga agaacatgct atccaatctc
3001 atctcttgta aatacatgtc cccctgtgag ttctgggctg atttgggtct ctcatacctc
3061 tgggaaacag accttttttct ctcttactgc ttcatgtaat tttgtatcac ctcttcacaa

FIG. 19A

3121 tttagttcgt acctggcttg aagctgctca ctgctcacac gctgcctcct cagcagcctc
3181 actgcatctt tctcttccca tgcaacaccc tcttctagtt accacggcaa cccctgcagc
3241 tcctctgcct ttgtgctctg ttcctgtcca gcaggggtct cccaacaagt gctctttcca
3301 ccccaaaggg gcctctcctt ttctccactg tcataatctc tttccatctt acttgccctt
3361 ctatactttc tcacatgtgg ctccccctga attttgcttc ctttgggagc tcattctttt
3421 cgccaaggct cacatgctcc ttgcctctgc tctgtgcact cacgctcagc acacatgcat
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3541 tgctttcttc atgttcagtc acatgtgctc tcgggtgccc tgcattcaca gctacgtgtg
3601 cccctctcat ggtcatgggt ctgcccttga gcgtgtttgg gtaggcatgt gcaatttgtc
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3901 ctccattgca ttcatgtact accctcagtc tacactcaca atcatcttct cccaagactg
3961 ctccctttttg ttttgtgttt ttttgagggg aattaaggaa aaataagtgg gggcaggttt
4021 ggagagctgc ttccagtgga tagttgatga gaatcctgac caaaggaagg cacccttgac
4081 tgttgggata gacagatgga cctatggggt gggaggtggt gtccctttca cactgtggtg
4141 tctcttgggg aaggatctcc ccgaatctca ataaaccagt gaacagtgtg actcggaaaa
4201 aaaaaaaaaa aaaaaaaaaaa

FIG.19B

PROXIMAL TO HSN-1. FCMD, DYS LOCI ON CHROMOSOME 9

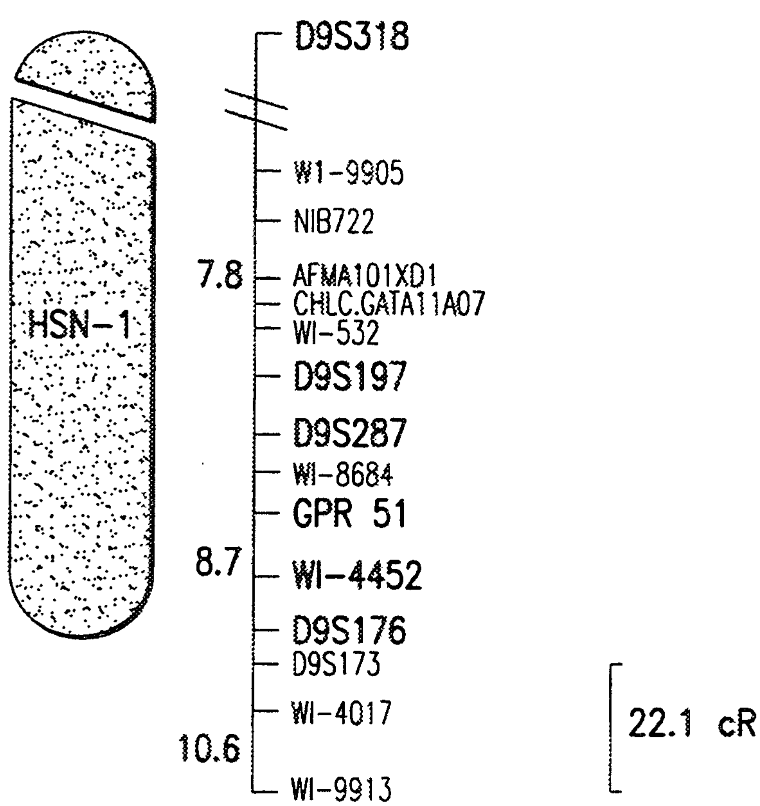

FIG. 20

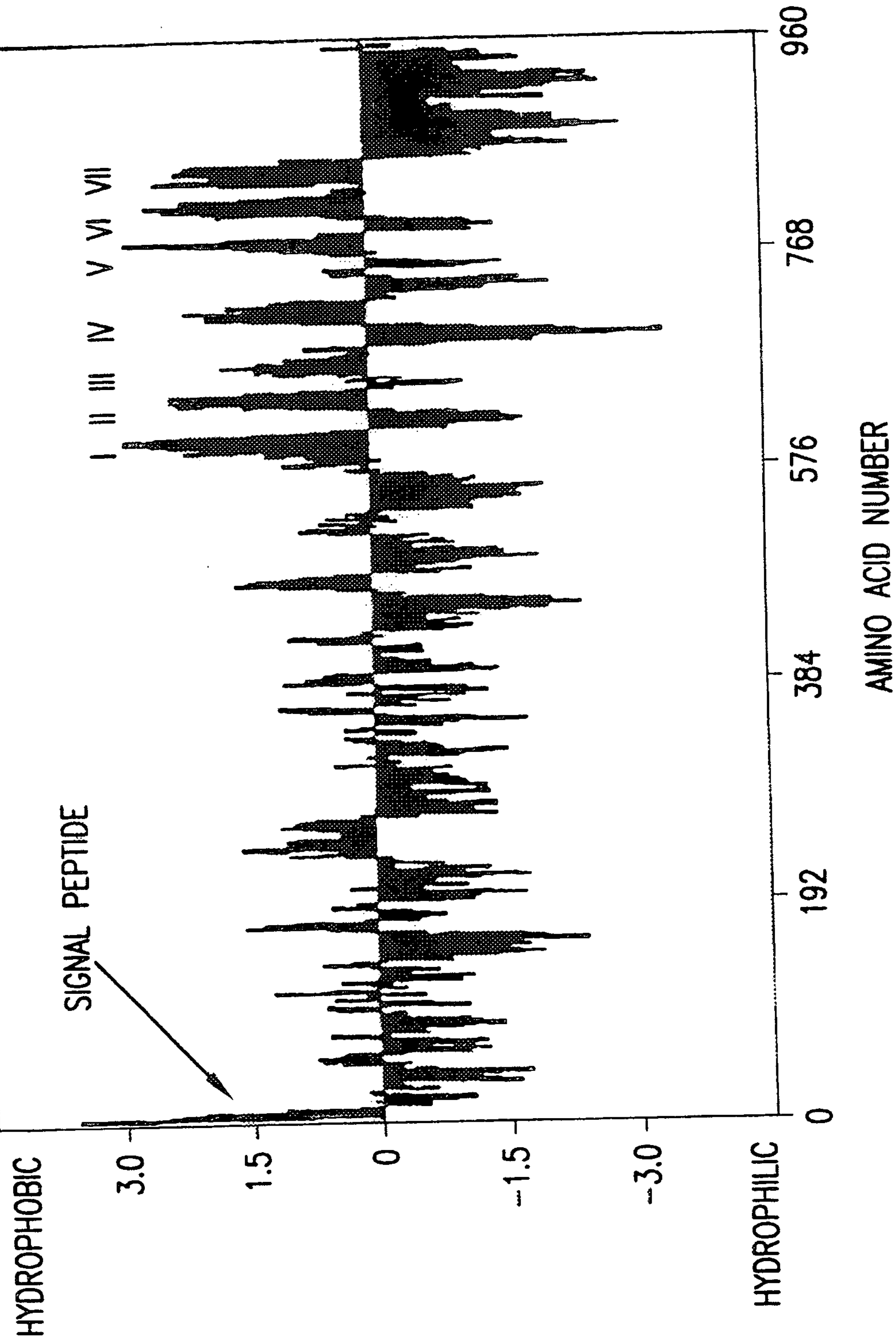
HYDROPHOBIC
3.0
1.5
0
-1.5
-3.0
HYDROPHILIC
SIGNAL PEPTIDE
I II III IV V VI VII
0
192
384
576
768
960
AMINO ACID NUMBER

FIG.21

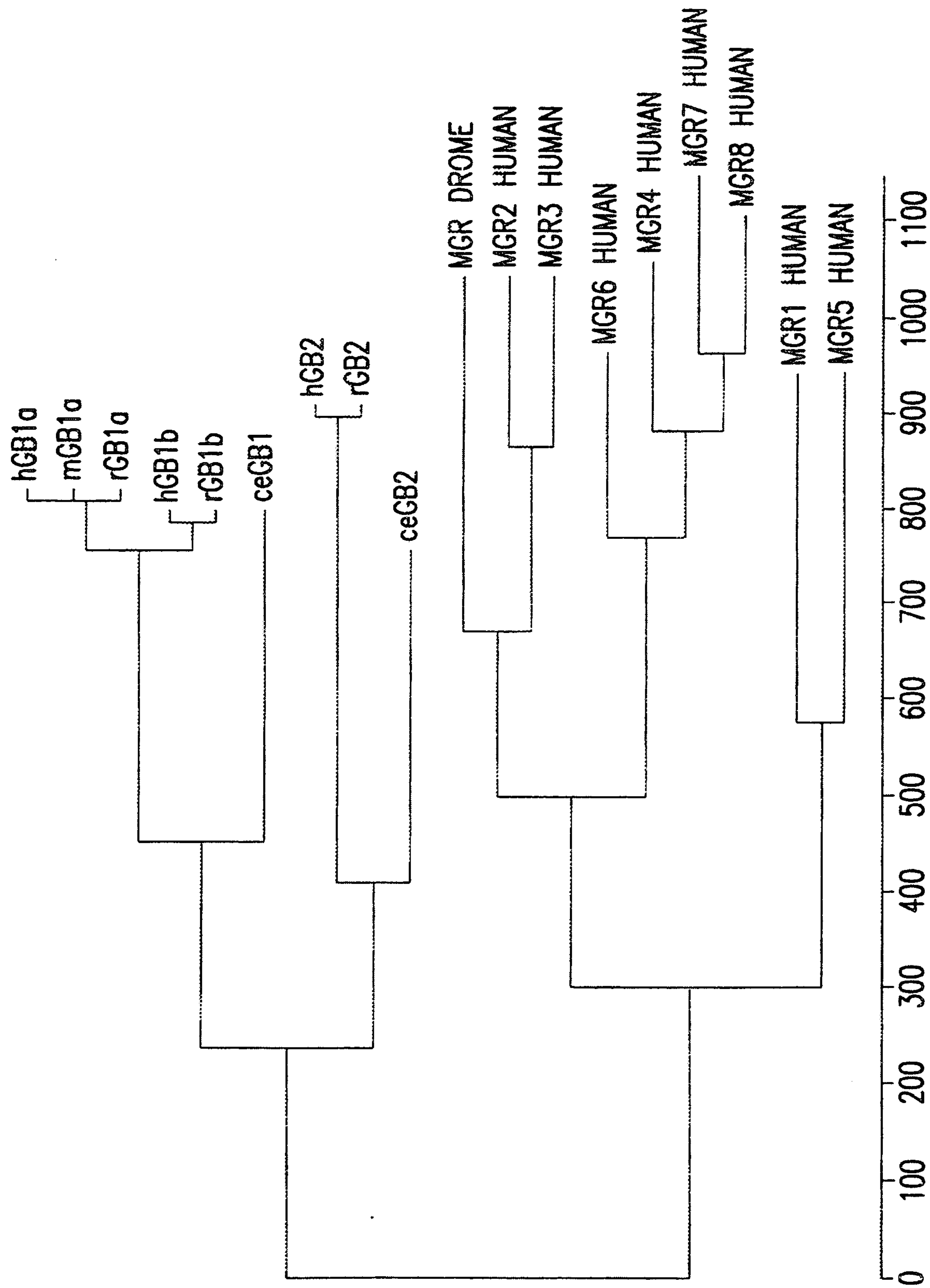
hGB1a
mGB1a
rGB1a
hGB1b
rGB1b
ceGB1
hGB2
rGB2
ceGB2
MGR DROME
MGR2 HUMAN
MGR3 HUMAN
MGR6 HUMAN
MGR4 HUMAN
MGR7 HUMAN
MGR8 HUMAN
MGR1 HUMAN
MGR5 HUMAN
0
100
200
300
400
500
600
700
800
900
1000
1100

FIG. 22

COILED-COIL DOMAIN IN C-TERMINUS OF gb1a AND HG20 MEDIATING HETERODIMERIZATION

```
gb1  ....WQSEA.QDTMKTGSSTNNNEEEK...SRLLEK..ENRELEKIIAEKEERVSELRHQLQSRQQLRSRRHPP
hg20 QNRRFQFTQNQKKEDSKTSTSVTSVNQASTSRLEGLQSENHRLRMKITELDKDLEEVTMQLQDTPEKTTYIKQN
```

FIG.23

```
                    10        20        30        40        50        60        70        80        90        100
Human GABA-B1aR     MLLLLLLAPLFLRPPGAGGAQTPNATSEGCQIIHPPWEGGIRYRGLTRDQVKAINFLPVDYEIEYVCRGEREVVGPKVRKCLANGSWTDMDTLSRCVRICSKSYLTL
C.elegans GABA-B1   ----------------------------------------------------------------------------------------------------------
C.elegans GABA-B2   ----------------------------------------------------------------------------------------------------------
Human GABA-B2       ----------------MASPRSSGQPGPPPPPPPP-----------------------------------------------------------------PA

110       120       130       140       150       160       170       180       190       200       210       220
ENGKVFLTGGDLPALDGARVDFRCDPDFHLVGSSRSICSQGQWSTPKPHCQVNRTPHSERRAVYIGALFPMSGGWPGGQACQPAVEMALEDVNSRRDILPDYELKLIHHDSKCDPGQ
-----MFVRSSWLLLWGTIVWASAEPVTLHIGGTFPMESGG-----------------------------SGGWAGGEACLPAVEMALKDVNSRLDILPGYMLNMTNHNSQCQPGL
----------------MNIFRRHGGIPLPLGVFTVQK-------------------------------------EGFPDALPAIRTALSHVHSRSCILQGYRLEMIVKDTHCKTSQ
RLLLLLLLPLLLPLAPGAWGWARGAPRPPPSSPPLSIMG------------------------LMPLTKEVAKGSIGRGVLPAVELAIEQIRNESLLRPYFLDLRLYDTECDNAK

230       240       250       260       270       280       290       300       310       320       330       340
ATKYLYELLYNDPIKIILMPG-CSSVSTLVAEAARMWNLIVLSYGSSSPALSNRQRFPTFFRTHPSATLHNPTRVKLFEKWGWKKIATIQQTTEVFTSTLDDLEERVKEAGIEITFR
AMQQLYDFLYKPPTKLMLLTG-CSPVTTVIAEAAPVWKLMVLSYGGSSPALSNRNRFPTLFRTHPSANMQNPTRIHIMEKFKWKRFTILMSVEEVFVTTAKDLEVSERKKGIKVD-R
GMKALFDLIIASRPRPVAIIGGQCTEVNEPIAMALKYWQIVQLSYAETHAMNGQLQLFTTFFRVVPGSRNTNMAKCKFVNHFGWKRVGTVKQNDQ--------------------
GLKAFYDAIKYGPNHLMVFGGVCPSVTSIIAESLQGWNLVQLSFAATTPVLADKKKYPYFFRTVPSDNAVNPAILKLLKHYQWKRVGTLTQDVQRFSEVRNDLTGVLYGEDIEISDT

350       360       370       380       390       400       410       420       430       440
QSFFSDPAVPVKNLKRQDARIIVGLFYETEARKVFCEVYKERLFGKKYVWFLIGWYADNWFKIYDP---SINCTVDEMTEAVEGHITTEIVMLNPAN-----TRSISNMTSQEFVEK
QSFYGDPTDAMKTLQRQDARIIVGLFYVTEARKVLCQAYHHGLYGRRYVWFFIGWYADTWYIPPP--EEHLNCTAEQMTEAAEYHFTTESVMLSRDN-----IPAISEMTGMQFQQR
---------PRYALVRGDVRIILVDVDEEMAATVLCAGYHRGMYGDNYVWILPGYHSDRWLNQ-----THDNCTVEEMREAAKNHFSVEFALTRRDVDTKIVGNTVSPYVTLNLFQR
ESFSNDPCTSVKKLKGNDVRIILGQFDQNMAAKVFCCAYEENMYGSKYQWIIPGWYEPSWWEQVHTEANSSRCLRKNLLAAMEGYIGVDFEPLSSKQ-----IKTISGKTPQQYERE

450       460       470       480       490       500       510       520       530       540       550       560
LTKRLKRHPEETGGFQEAPLAYDAIWALALALNKTSGGGGRS--GVRLEDFNYNNQTITDQIYRAMNSSSFEGVSGHVVFDASGSRMAWTLIEQLQGGSYKKIGYYDSTKDDLSWSK
LTQYFQKDTANVGGFPEAPLAYDAVWALALAFNCTRNN-LPS--HIRLENFTYDNKVIADTLFQCVKNTSFRGVSGKVMFSDSGDRIARTQIEQMQGGKYKIMGYYDTTSGDLEWYN
AGDVWNEITQLDPNNTWRGYLYDGLWTLAIALSHSMGD--------------NAEFSHHKMMEAIDNSSFQGLTGKVKFANN-ERLGLVDIKQWSDGQYVPFAVYDG--ADDEFKI
YNNKR---SGVGPS-KFHGYAYDGIWVIAKTLQRAMETLHASSRHQRIQDFNYTDHTLGRIILNAMNETNFFGVTGQVMFRNG-ERMGTIKFTQFQDSREVKVGEYNAVADTLEIIN
```

FIG.24A

```
570        580        590    TMS-1  610        620        630      TMS-2  650        660      TMS-3
TDKMIGG-SPPADQTLVIKTFR-FLSQKLFISVSVLSSLGIVLAVVCLSFNIYNSHVRYIQNSQPNLNNLTAVGCSLALAAVFPLGLDG--YHIGRNQFPFVCQARLWLLGLGFSLG
KEQWLNGKGPPPD-STVIKTFN-SYSDFLIFSSTILQYFSQFLALLHVSSFTFLHKNIIFQ-SQPECNNILLIGCSLCLFSLFLIGLPSDDISISESLFPLLCHARVTILLFGFTFA
IDSTTKGWSPPLD-STITERRREHISSILFLAMSLLALIGIFLALIFLLINFRYRNHRFIKMSSPNLNNIIIAGSICTFASVIMLGLDT--RIVSPDVFVWLCYTKTWILCIGFTLS
DTIRFQQSEPPKDKTIILEQLR-KISLPLYSILSALTILGMIMASAFLFFNIKNRNQKLIKMSSPYMNNLIILGGMLSYASIFLFGLDG--SFVSEKTEETLCTVRTWILTVGYTTA

680        690        700        710        720  TMS-4 730        740        750        760      TMS-5      790
                                                                                     C
YGSMFTKIWWVHTVFTKKEEKKEWRKTLEPWKLYATVGLLVGMDVLTLAIWQIVDPLHRTIETFAKEEPKEDIDVSILPQLEHCSSRKMNTWLGIFYGYKGLLLLLGIFLAYETKSV
YGSMFAKMWIVHRMGATENQQLASRQPISSSKFYVIMAALTAVDVFVCFVWVLIDPLHLTEQKFPLFADSE-EDEMIMPVLQQCQSNQQEVWIGIIMGFKCLLLVFGTFLSYETRNL
FGAMFSKTWRVHSIFTN---IRMDRKAIKDSKLFIILGILLFIDICVLVTWAFVSPFSYTVEQFKFLIFSARRNIVIIPEVEKCNSSHSGVFQAVLYAVKGVLMILGCFLAWETRHV
FGAMFAKTWRVHAIFKN---VKMKKKIIKDQKLLVIVGGMLLIDLCILIQWQAVDPLRRTVEKYSMEPDPAGRDISIRPLLEHCENTHMTIWLGIVYAYKGLLMLFGCFLAWETRNV

800        810  TMS-6          830     TMS-7  850        860                   870        880        890
STEKINDHRAVGMAIYNVAVLCLITAPVTMILSS-QQDAAFAFASLA-IVFSSYITLVVLFVPKMRRLIITRGEWQS-------------EAQDTMKTGSSTNNNEEEKSRLLEKENR
KLRFINDSRFVGLAIYNVAVMTLVTAPVTLLIHGKVDANFAFISLTSVLICTYISVGLIYGPKIRHIIKVPPSAD-------------EIQLNGNVGPGVMSKVDQK---------
NVPALNDSKYIGTSVYCCVVMSVLGLSTSVILQE-RVNEMFSLASFF-VIFSTTLTLCLVFVPKVRFLELCCIGS------------------------------------------
SIPALNDSKYIGMSVYNVGIMCIIGAAVSFLTRD-QPNVQFCIVALV-IIFCSTIITLCLVFVPKLITLRTNPDAATQNRRFQFTQNQKKEDSKTSTSVTSVNQASTSRLEGLQSENH

900        910        920        930        940                                           950        960
ELEKIIAEKEERVSELRHQLQSRQQLRSRRHPPTPPEPSGGLPRG-------------------------------------------PPEPPDRLSCDGSRVHLLYK---------
----------RYDMLKKE--------------------------------------------------------------------------------------------------
--------------------------------------------------------------------------------------------------------------------
RLRMKITELDKDLEEVTMQLQDTPEKTTYIKQNHYQELNDILNLGNFTESTDGGKAILKNHLDQNPQLQWNTTEPSRTCKDPIEDINSPEHIQRRLSLQLPILHHAYLPSIGGVDAS

----------------------------
----------------------------
CVSPCVSPTASPRHRHVPPSFRVMVSGL
```

FIG.24B

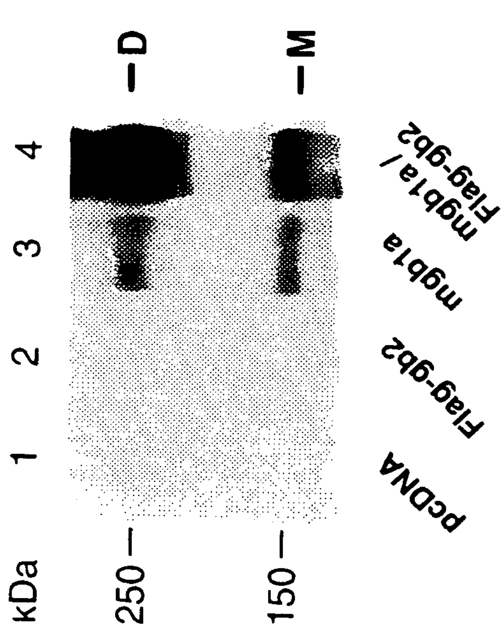

FIG.25A

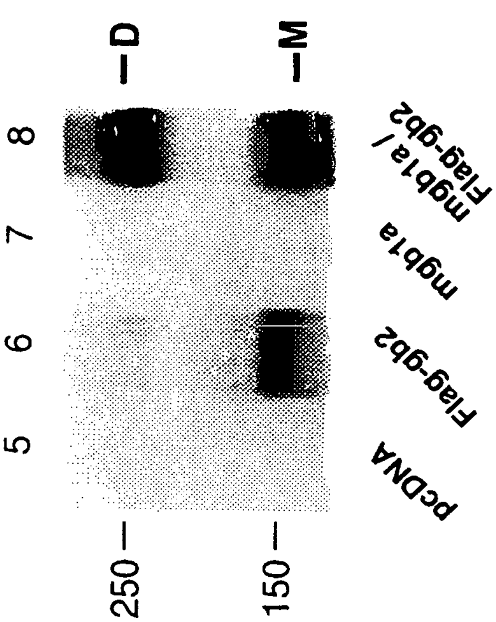

FIG.25B

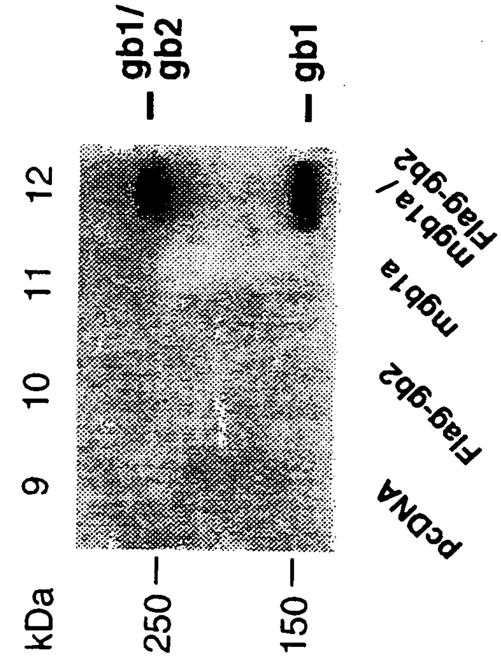

FIG.25C

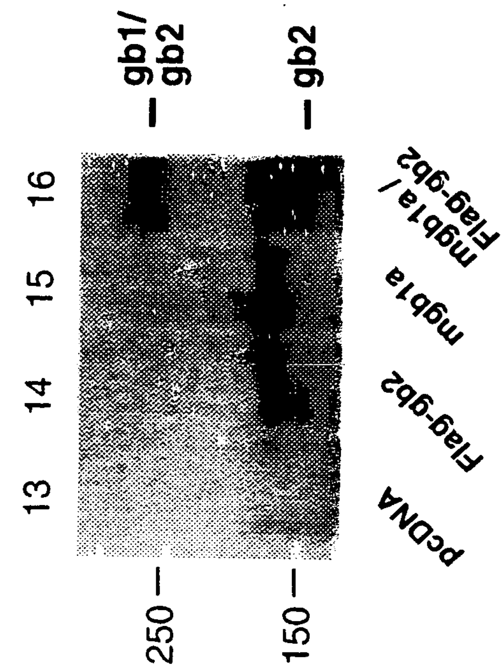

FIG.25D

```
                                          SCR1                                                                                      SCR2
mGABAb1a MLLLLLL.LFLRPLGAGGAQTPNVTSEGCQIIHPPWEGGIRYRGLIRDQVKAINFLPVDYEIEYVCRGEREVVGPKVRKCLANGSWTDMDTPSRCVRICSKSYLTLENGKVFLTGGDLP:119
hGABAb1a MLLLLLLAPLFLRPPGAGGAQTPNATSEGCQIIHPPWEGGIRYRGLTRDQVKAINFLPVDYEIEYVCRGEREVVGPKVRKCLANGSWTDMDTPSRCVRICSKSYLTLENGKVFLTGGDLP:120
hGABAb2  M..............AS..............PRRSG.......................QPGRPPPPPP.............PPAR.........LLL....LLL....LP: 30
mGluR1   MVGLLLFFFPAIF.LEVS.............LLPRSPGR...........................................................KVLLAGASSQRSVARMDGD: 44
LivK     MKRNAKTIIAGMIALAIS.............HTAMADDI...........................................................K...........VA.....: 29
livBP    ...............................EDI...........................................................K...........VA.....:  6

                                                                    SCR3
mGABAb1a ALDGARVDFRCDPDFHLVGSSRSICSQGQWSTPKPHCQVNRTPHSERRAVYIGALFPMS..GGWPG.GQACQPAVEMALEDVNSRRD.ILPDYELKLIHHD..SKCDPGQATKYLYELLY:233
hGABAb1a ALDGARVDFRCDPDFHLVGSSRSICSQGQWSTPKPHCQVNRTPHSERRAVYIGALFPMS..GGWPG.GQACQPAVEMALEDVNSRRD.ILPDYELKLIHHD..SKCDPGQATKYLYELLY:234
hGABAb2  LL...........LPLAPGAWGWA....RGAPRP.......PPSSP.PLSIMGLMPLTKEVAKGSIGRGVLPAVELAIEQI..RNESLLRPYFLDLRLYD..TECDNAKGLKAFYDAIK:122
mGluR1   VIIGALFSVHHQPPAEKVPERKCGEIREQYGIQR..................VEAMFHTLDKINADPVLLPNITLGSE..IRD.SCWHSSVALEQSIEFIRDSLISIRDEKDGI...NRCLP:142
LivK     .VVGAM..............SGPI.AQWGIME..................FNGAEQAIKDINAKGGIKGDKLVGVE..YDD.AC...........................DPKQAVAVANKIVN: 90
livBP    .VVGAM..............SGPV.AQYGDQE..................FTGAEQAVADINAKGGIKGNKLQIAK..YDD.AC...........................DPKQAVAVANKVVN: 67

mGABAb1a ND..........PIKIILMPG.CSSVSTLVAEAARMWN...LIVLSYGSSSPALSNRQRFPTFFRTHPSATLHNPTRVK.LFE...KWGWKKIATIQQTTEVFTS.TLDDLEERVKEAGI:334
hGABAb1a ND..........PIKIILMPG.CSSVSTLVAEAARMWN...LIVLSTGSSSPALSNRQRFPTFFRTHPSATLHNPTRVK.LFE...KWGWKKIATIQQTTEVFTS.TLDDLEERVKEAGI:335
hGABAb2  YG..........PNHLMVFGGVCPSVTSIIAESLQGWN...LVQLSFAATTPVLADKKKYPYFFRTVPSDNAVNPAILK.LLK...HYQWKRVGTLTQDVQRFSE.VRNDLTGVLYGEDI:224
mGluR1   DGQSLPPGRTKKPIAGVI.GPGSSSVAIQVQNLLQLFD...IPQIAYSATSIDLSDKTLYKYFLRVVPSDTLQ....ARAMLDIVKRYNWTYVSAV.HTEGNYGESGMDAFKELAAQEGL:253
LivK     DG...........IKYVI.GHLCSS.STQPAS..DIYEDEGILMISPGATAPELTQRG.YQHIMRTAGLDSSQGPTAAKYILETVKP.QR..IAII.HDKQQYGE.GLAR....SVQDGL:185
livBP    DG...........IKYVI.GHLCSS.STQPAS..DIYEDEGILMITPAATAPELTARG.YQLILRTTGLDSDQGPTAAKYILEKVKP.QR..IAIV.HDKQQYGE.GLAR....AVQDGL:162
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FIG.26A-1

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mGABAb1a EIITFRQSFFSDPAVPVKN.....LKRQDARI....IVGLFYET...EARKVFCEVYKERLFGKKYVWFLIGWYADNWFK......TYDPSINCTVEEM.TEAVEGHITTE....IVMLNP:431
hGABAb1a EIITFRQSFFSDPAVPVKN.....LKRQDARI....IVGLFYET...EARKVFCEVYKERLFGKKYVWFLIGWYADNWFK......IYDPSINCTVDEM.TEAVEGHITTE....IVMLNP:432
hGABAb2  EIISDTESFSNDPCTSVKK.....LKGNDVRI....ILGQFDQN...MAAKVFCCAYEENMYGSKYQWIIPGWYEPSWWEQ...VHTEANSSRCLRKNL.LAAMEGYIGVD....FEPLSS:324
mGluR1   CIAHSDKIYSN...AGEKSFDRLLRKLRERLPKARVVVCFCEGM..TVRGLLSAM...RRLGVVGEFSLIG..SDGWADRDEVIEGYEVEANGGIT.IKLQSPEVRSFDDYFLKLRLDTN:362
LivK     KAANANVVFFDGITAGEKDFSALI....ARLKKENIDFVYYGGYYPEMGQMLRQA...RSVGLKTQF..MG..PEG............V.GNASLSNIAGDAAEG.........M.LVTM:271
livBP    KKGNANVVFFDGITAGEKDFSTLV....ARLKKENIDFVYYGGYHPEMGQILRQA...RAAGLKTQF..MG..PEG............V.ANVSLSNIAGESAEG.........L.LVTK:248

mGABAb1a ANTRSISNMTSQEFVEKLTKRLKRHPE......ETGGF.....QEAPLAYD..AIWALALALNK...TSGGGRSGVRLEDFNYNNQTITDQIYRAMNSSSFEGVSGHVV.FDASGSRMA:534
hGABAb1a ANTRSISNMTSQEFVEKLTKRLKRHPE......ETGGF.....QEAPLAYD..AIWALALALNK...TSGGGRSGVRLEDFNYNNQTITDQIYRAMNSSSFEGVSGHVV.FDASGSRMA:535
hGABAb2  KQIKTISGKTPQQYEREYNN..KRSGV......GPSKF.....HG..YAYD..GIWVIAKTLQRAMETLHASSRHQ.RIQDFNYTDHTLGRIILNAMNETNFFGVTGQVV.FR.NGERMG:424
mGluR1   TRNPWFPEFWQHRFQCRLPGHLLENPNFKRICTGNESLEENYVQDSKMGFVINAIYAMAHGLQ.NMHHALCPGHVGL.CDAMKPID...GSKLLDFLIKSSFIGVSGEEVWFDEKGDAPG:477
LivK     PK..........RYD........QDPANQGIV...DALKAD.KKDPSGPYV.WITYAAVQSLATALERTGSDEPLAL.VKDLKANG...ANTVIGPLN............WDEKGDLKG:351
livBP    PK..........NYD........QVPANKPIV...DAIKAK.KQDPSGAFV.WTTYAALQSLQAGLNQ..SDDPAEI.AKYLKANS...VDTVMGPLT............WDEKGDLKG:326

mGABAb1a WTL.IE.........QLQGGSYKKIGYYD.................STKDDLS.WSKTDKWIGGS....PPAD.............................:575
hGABAb1a WTL.IE.........QLQGGSYKKIGYYD.................STKDDLS.WSKTDKWIGGS....PPA..............................:575
hGABAb2  .TIKFT.........QFQDSREVKVGEYN.................AVADTLEIINDTIRFQGSE....PPKDKTIILEQLR....................:475
mGluR1   RYDIMNLQYTEANRYDYVHVGTWHEGVLNIDDYKIQMNKSGVVRSVCESPCLKGQIKVIRKGEVSCCWICTACKENEYVQDEFTCKACDLGWPPNADLTGCEPIPVRY:585
LivK     ..............FDF.......GVFQ.............................WHADGS....STAAK............................:369
livBP    ..............FEF.......GVFD.............................WHANGT....ATDAK............................:344
```

FIG.26A-2

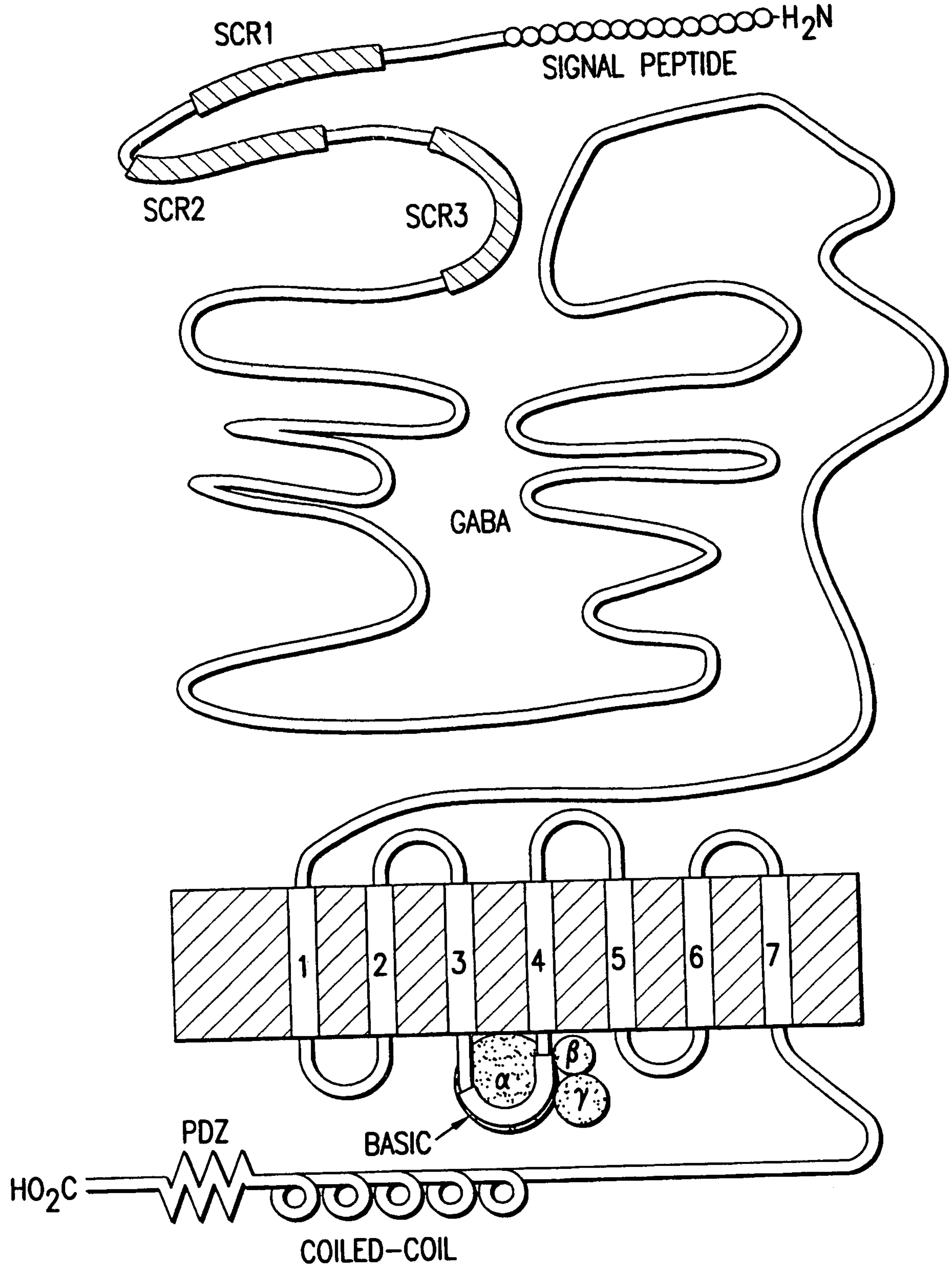
SCR1
H2N
SIGNAL PEPTIDE
SCR2
SCR3
GABA
1
2
3
4
5
6
7
β
α
γ
PDZ
BASIC
HO2C
COILED-COIL

FIG.26B

```
                 COILED-COIL DOMAIN
mGABAb1a  .KMR...RLITRGEW....QSETQDTMKTGSS.TNNNEEEKSRL..LEKE:  39
hGABAb1a  PKMR...RLITRGEW....QSEAQDTMKTGSS.TNNNEEEKSRL..LEKE:  40
hGABAb2   ITLRTNPDAATQNRRFQFTQNQKKEDSKTSTSVTSVNQASTSRLEGLQSE:  50

mGABAb1a  NR.......ELEKIIAE...............KEERVSE............:  56
hGABAb1a  NR.......ELEKIIAE...............KEERVSE............:  57
hGABAb2   NHRLRMKITELDKDLEEVTMQLQDTPEKTTYIKQNHYQELNDILNLGNFT: 100

mGABAb1a  .........LRHQLQSRQQL.RSRRHPP.TPPDPSGGLPRGPSEPPDRLS:  95
hGABAb1a  .........LRHQLQSRQQL.RSRRHPP.TPPEPSGGLPRGPPEPPDRLS:  96
hGABAb2   ESTDGGKAILKNHLDQNPQLQWNTTEPSRTCKDPIEDI.NSPEHIQRRLS: 149

                          PDZ
mGABAb1a  ................CDGSRV.............HLL..YK......: 106
hGABAb1a  ................CDGSRV.............HLL..YK......: 107
hGABAb2   LQLPILHHAYLPSIGGVDASCVSPCVSPTASPRHRHVPPSFRVMVSGL: 197
```

FIG.27

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PCT

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, DZ, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR (utility model), KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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Published:
— *With international search report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/14122 A1

(54) Title: CONTAINER PRODUCTION PROCESS

(57) Abstract: The invention provides the use of bimodal HDPE in the production by blow moulding of medium to large volume containers having improved environmental stress crack resistance.

Container Production Process

This invention relates to improvements in and relating to the production of containers by blow moulding of a polymer material, in particular a high density polyethylene (HDPE).

In the production of containers using polymer materials, a variety of moulding techniques may be used, in particular blow moulding, slush moulding, injection moulding and rotational moulding. For very large containers, e.g. for use as oil or water tanks, rotational moulding is a preferred technique; however for medium to large sized containers, e.g. 8 to 250L volume, blow moulding is commonly used.

Generally in the blow moulding of containers a heated parison or extrudate is expanded to contact the interior surface of a mould by the action of pressurized gas within the parison and/or by application of a partial vacuum within the mould, e.g. by use of a mould having within its surface gas outlet vents through which a partial vacuum may be applied.

For blow moulding to be successful, the heated polymer must be able to stretch smoothly to produce a satisactorily uniform skin in contact with the mould surface.

In the production of containers by blow moulding of polyethylene, the polymer used has typically been a high molecular weight (HMW) high density polyethylene (HDPE), generally with a melt flow rate (MFR_{21}) in the range 2 to 12 g/10 min. Such materials may be produced for example by chromium-catalysed ethylene polymerization. However the choice of such materials essentially represents a compromise between the properties of the polymer, in particular the processability properties required for the blow moulding to be performed efficiently and successfully and the mechanical and chemical properties

required for the end uses of the blow moulded containers, e.g. impact resistance, stiffness, and environmental stress crack resistance (ESCR).

We have now found that large volume blow moulded containers which have excellent ESCR properties may be produced if one uses a high density polyethylene (HDPE) comprising at least two polyethylene components having different molecular weight distributions wherein at least one of said components is an ethylene copolymer. Such HDPE is referred to hereinafter as bimodal.

Thus viewed from one aspect the invention provides a process for the preparation of an at least 2L (e.g. 2 to 1000L, preferably 2 to 250L, more preferably 8 to 230L, still more preferably 30 to 225L) volume polyethylene container which process comprises blow moulding a bimodal HDPE.

In the process of the invention, the parison is conveniently part-blown before the mould is closed, the mould is then closed and the blowing of the parison is completed.

By polyethylene is meant a polymer the majority by weight of which derives from ethylene monomer units. While the different polyethylene components of the bimodal HDPE may all be ethylene copolymers, and while an ethylene homopolymer may be one of the components, the polyethylene components cannot all be ethylene homopolymers. Where one component is an ethylene homopolymer, this is preferably the component with the lower weight average molecular weight (Mw) and its Mw is preferably 5000 to 100000 D, more preferably 20000 to 40000 D. In the ethylene copolymer components, comonomer contributions, e.g. up to 20% by weight more preferably up to 10% by weight, may derive from other copolymerizable monomers, generally C_{3-20}, especially C_{3-10}, comonomers, particularly singly or multiply ethylenically unsaturated comonomers, in particular C_{3-10} α-olefins such as propene, but-1-ene, hex-1-ene, etc.

(The term ethylene copolymer is used herein to relate to a polyethylene deriving from ethylene and one or more such copolymerizable comonomers). Moreover, the polyethylene may contain minor, e.g up to 10% by weight, preferably up to 5% by weight of other polymers, e.g. other polyolefins in particular polypropylenes, as well as additives such as plasticizers, colours, fillers, radiation stabilizers, antioxidants, etc., generally in amounts up to 10% by weight, preferably up to 5% by weight.

By HDPE is meant a polyethylene having a density of 940 to 980 kg/m^3, preferably 945 to 975 kg/m^3, more preferably 945 to 960 kg/m^3, and a crystallinity of 60 to 95%, preferably 70 to 90%.

The HDPE used according to the invention is a bimodal or multimodal polymer. By bimodal (or multimodal), it is generally meant that the polymer consists of at least two fractions (components), one of which has a relatively low molecular weight and a relatively high density and another of which has a relatively high molecular weight and a relatively low density. Typically the molecular weight distribution (MWD) of a polymer produced in a single polymerization stage using a single monomer mixture, a single polymerization catalyst and a single set of process conditions (ie. temperature, pressure etc.) will show a single maximum, the breadth of which will depend on catalyst choice, reactor choice, process conditions, etc, ie. such a polymer will be monomodal.

A bimodal or multimodal polyethylene may be produced by blending two or more monomodal polyethylenes having differently centred maxima in their MWDs. Alternatively and preferably the bimodal polyethylene may be produced by polymerization using conditions which create a bimodal or multimodal polymer product, e.g. using a catalyst system or mixture with two or more different catalytic sites, using two or more stage

polymerisation process with different process conditions in the different stages (e.g. different temperatures, pressures, polymerisation media, hydrogen partial pressures, etc). (See EP-A-778289)

Such a bimodal (or multimodal) HDPE may be produced relatively simply by a multistage ethylene polymerization, e.g. using a series of reactors, with comonomer addition in only the reactor(s) used for production of the higher/highest molecular weight component(s). Examples of bimodal PE production are given in EP-A-778289 and WO92/12182.

If an ethylene homopolymer component is produced by slurry polymerization involving use of recycled diluent, that diluent may contain small amounts of higher α-olefins as contaminants. Likewise where an earlier polymerization stage has produced an ethylene copolymer component, small amounts of comonomer may be carried over to an ethylene homopolymerization stage. Accordingly, by ethylene homopolymer is meant herein a polymer containing at least 99.9% by weight of ethylene units. Likewise as in a multistage/multireactor polymerization using more than one catalyst system, the homopolymerization catalysts may be at least partially active during the copolymerization reaction, any copolymer component making up less than 5% by weight of the total polymer shall not be considered to be the lowest molecular weight component in an HDPE according to the invention.

The copolymer component(s) of the HDPE used according to the invention will generally contain at least 0.1% by weight, preferably at least 0.5% by weight of non-ethylene monomer units, e.g. 0.5 to 6% of such comonomer units.

The polymerization reactions used to produce the HDPE of the invention may involve conventional ethylene homopolymerization or copolymerization reactions, e.g. gas-phase, slurry phase, liquid phase polymerizations,

using conventional reactors, e.g. loop reactors, gas phase reactors, batch reactors etc. (see for example WO97/44371 and WO96/18662). The catalyst systems used may likewise be any conventional systems, e.g. chromium catalysts, Ziegler-Natta and metallocene or metallocene:aluminoxane catalysts, either homogeneous or more preferably heterogeneous catalysts, e.g. catalysts supported on inorganic or organic particulates, in particular on magnesium halides or inorganic oxides such as silica, alumina or silica-alumina. For the preparation of the high molecular weight component in particular it is especially desirable to use supported Ziegler-Natta catalysts as the molecular weight can then conveniently be controlled using hydrogen. It is also possible to use supported metallocene catalysts as it is particularly straightforward to select desired molecular weights by appropriate selection of particular metallocenes. The metallocenes used will typically be group IVa to VIa metals (in particular Zr or Hf) complexed by optionally substituted cyclopentadienyl groups, e.g. groups carrying pendant or fused substituents optionally linked together by bridging groups. Suitable metallocenes and aluminoxane cocatalysts are widely described in the literature, e.g. the patent publications of Borealis, Hoechst, Exxon, etc.

Typically however the HDPE will be prepared using multistage polymerization using a single catalyst system or a plurality of catalyst systems, e.g. two or more metallocenes, one or more metallocenes and one or more Ziegler-Natta catalysts, two or more chromium catalysts, one or more chromium catalysts and one or more Ziegler-Natta catalysts, etc.

The use of bimodal HDPE in the production of relatively large, ie. 2 to 1000L, e.g. 2 to 250L, preferably 8 to 240L, more preferably 20 to 230L, and still more preferably 30 to 225L, containers results in

surprisingly increased values for ESCR. Thus for example tests comparing containers made using conventional monomodal HDPE with bimodal HDPE have shown an increase in ESCR F_{50} from 250 hours to over 1000 hours. (ESCR F_{50} is measured as the time taken for 50% of samples to be broken when bent and contained in a detergent solution. The test is a standard one categorised as ESCR, ASTM D-1693 condition B, 10% Igepal). Such F_{50} values for relatively large containers are particularly surprising in view of the relatively low F_{50} values (ca. 400 h) achieved for small (0.45L) containers using blow-moulding of bimodal HDPE in EP-A-778289.

The bimodal HDPE used in the process of the invention preferably has the following characteristics
Density: 940 to 970, preferably 945 to 960 kg/m^3;
Weight Average Molecular Weight (Mw): 200000 to 450000, preferably 250000 to 350000 Dalton;
Number Average Molecular Weight (Mn): 6000 to 20000, preferably 7000 to 18000 Dalton;
MFR_{21}: 2 to 12 g/10 min, preferably 3 to 8 g/10 min;
Molecular Weight Distribution (i.e. Mw/Mn): 15 to 55, preferably 18 to 50;
Tensile Modulus: at least 900 mPa;
Comonomer Content: 0.5 to 10% wt, preferably 1 to 2 wt %.

MFR_2 and MFR_{21} are routinely measured according to ISO 1133 at 190°C under 2.16 and 21.6 kg loads respectively. Tensile modulus may be measured according to ISO 527.2.

Bimodal HDPE having such characteristics may be prepared by the technique described in EP-B-517868 or by conventional use of Ziegler Natta, metallocene or chromium catalysts. Especially preferably the bimodal HDPE is produced using a series of two or more reactors, in particular a loop reactor followed by one or more gas phase reactors, preferably using a Ziegler-Natta

polymerization catalyst.

The polymer used according to the invention preferably contains a high molecular weight component (e.g. with a molecular weight tail of about 5000000 Daltons) to increase melt strength of the HDPE composition under elongational deformation. This can be achieved in one of several ways, e.g.

(i) produce the HDPE in a multistage polymerization in which reactor conditions are set such that a very high molecular weight component is produced in one stage, e.g. by performing one reaction stage in the absence of chain terminating hydrogen, by selection of a catalyst system which produces a high molecular weight polyethylene, by use of polyenic comonomers (e.g. dienes) which produce long chain branching or otherwise promote production of a high molecular weight fraction; or

(ii) crosslinking of a polymer by timed addition of crosslinking agents in the extruder whereby to tailor-make a high molecular weight grade (i.e. to add a HMW "tail" to the polymer).

Viewed from a further aspect the invention provides a blow-moulded binded HDPE container having a volume of at least 2L (preferably 8 to 240L, etc), and an ESCR F_{50} of at least 500 hours, preferably at least 800 hours, more preferably 1000 hours, e.g. 1000 to 2000 hours.

The containers of the invention may be produced in conventional blow-moulding apparatus.

It will be appreciated therefore that the containers of the invention may be produced without loss of the impact strength or ESCR properties using faster than normal blowing rates, ie. with more efficient and economical usage of the blow moulding apparatus.

The containers of the invention may be used indoors or out of doors for storing gases, liquids or solids, especially liquids such as water, glues, solvent-based soaps, paints, varnishes, chemical solutions, oils and

other liquid chemicals. The containers may be open (e.g. tanks, basins, etc) or may be closed, for example with lids or caps or inlet or outlet ducts.

The invention will now be described further with reference to the following non-limiting Examples.

EXAMPLE 1

Catalyst Preparation

8.6g (66.4 mmol) of 2-ethyl-1-hexanol was added slowly to 27.8g (33.2 mmol) of 19.9% butyl-octyl-magnesium. The temperature was kept below 35°C

3.7g (1.0 mmol/g carrier) of 20% EADC (ethyl aluminium dichloride) was added to 5.9g of Sylopol 5510 silica/$MgCl_2$ carrier and the mixture was stirred for 1 hour at 30°C. 5.7g (0.9 mmol/g carrier) of the 2-ethyl-1-hexanol/butyl-octyl-magnesium complex was added and the mixture was stirred for 5 hours at 45°C. 0.6g (0.55 mmol/g carrier) of $TiCl_4$ was added and the mixture was stirred for 5 hours at 45°C. The catalyst was dried at 45-80°C for 3 hours.

EXAMPLE 2

Bimodal HDPE Polymer Preparation

Ethylene, propane and hydrogen were introduced at 2.4 kg/hour, 25 kg/hour and 1 g/hour respectively into a 50 dm^3 slurry loop reactor operated at 85°C and 65 bar. The catalyst of Example 1 was introduced at a rate sufficient to produce polyethylene (PE) at about 1.9 kg PE/hour. The MFR_2 and density of the PE product were estimated to be about 10 g/10 min and 970 kg/m^3 respectively. The slurry was withdrawn continuously and

introduced into a second loop reactor of volume 500 dm^3, operating at 95°C and 61 bar. Additional ethylene, propane and hydrogen were added to produce a PE of MFR_2 330 g/10 min and density 974 kg/m^3 at 30 kg PE/hour. The polymer, which contained the active catalyst, was continuously withdrawn, separated from the reaction medium and transferred to a gas phase reactor. Here additional ethylene, hydrogen and 1-butene comonomer were added to produce a PE of MFR_{21} 7.3 g/10 min and density 949 kg/m^3 at 69 kg PE/hour. The low MW (high MFR) fraction thus represented 45% of the total polymer.

EXAMPLE 3

Bimodal HDPE Polymer Production

Polymerization was effected as in Example 2 except that 36 kg PE/hour was formed in the second loop reactor, 77 kg PE/hour was formed in the gas phase reactor and the MFR_{21} and density of the final PE were 3.3 g/10 min and 952 kg/m^3 respectively. The low MW (high MFR) fraction represented 48% of the total polymer.

EXAMPLE 4

Bimodal HDPE Polymer Production

Ethylene, propane and hydrogen were introduced into a 50 dm^3 slurry loop reactor operating at 80°C and 65 bar at 2.0 kg/hour, 28 kg/hour and 1 g/hour respectively. A catalyst according to Example 3 of EP-B-688794 was added to produce PE at a rate of 1.6 kg PE/hour. The MFR_2 and density of the PE were estimated to be about 10 g/10 min and 970 kg/m^3 respectively. The slurry was continuously withdrawn and introduced into a second loop reactor of 500 dm^3 volume operating at 95°C and 61 bar. Additional ethylene, propane and hydrogen were added so that the

second loop reactor produced PE at a rate of 34 kg PE/hour with MFR_2 and density of 55 g/10 min and 970 kg/m^3 respectively. PE polymer, containing the active catalyst, was withdrawn continuously from the second loop reactor, separated from the reaction medium and transferred to a gas phase reeactor where additional ethylene, hydrogen and 1-butene comonomer were added such that PE was produced at 70 kg PE/hour with MFR_{21} and density of 6.9 g/10 min and 948 kg/m^3 respectively. The fraction of low MW (high MFR) polymer was thus 50%.

EXAMPLE 5

Bimodal HDPE Polymer Production

Polymerization was effected as in Example 4 except that 38 kg PE/hour and 78 kg PE/hour were formed in the second loop reactor and the gas phase reactor respectively. The final PE had MFR_{21} and density of 7.3 g/10 min and 948 kg/m^3 respectively and the low MW fraction was 50%.

EXAMPLE 6

Blow Moulding

30L containers were blow moulded on a Krupp Kautex KB50 blow moulding machine at an extruder temperature of 210°C using the HDPE polymers of Examples 2 to 5. The containers were pre-stored at 42°C for 3 weeks containing n-butyl acetate. The n-butyl acetate was replaced with ethylene glycol and the containers were cooled to -20°C within 24 hours and drop-tested at -20°C using the test method of ASTM 2463-90, giving values of 2 to 3.25 m. The ESCR F_{50} values were also determined using ASTM D1693, condition B.

EXAMPLE 7

Polymer Properties

The measured properties of the polymers of Examples 2 to 5 and a comparison PE prepared using a chromium based catalyst are set out in Table 1 below. The comparison PE is commercially available as HE8214 from Borealis AS.

Property	Example 2	Example 3	Example 4	Example 5	HE 8214
MFR_{21} (g/10 min)	7.3	3.3	6.9	7.3	2.7
Density (kg/m^3)*	948.5	951.5	947.9	948	950.4
Mw	260000	370000	305000	316000	345000
Mn	8300	7700	12000	16000	16000
MWD	31	48	25	20	22
Tensile Modulus (mPa)†	920	1030	920	910	1065
ESCR F50 (hours)	542	>1000	>1000	>1000	391
Comonomer	1-butene	1-butene	1-butene	1-butene	1-hexene
Comonomer content (% wt)	1.8	1.1	1.4	1.2	<0.1

* ISO 1183

† ISO 527-2

Claims

1. A process for the preparation of at least 2L volume polyethylene container which process comprises blow moulding a bimodal HDPE.

2. A process as claimed in claim 1 wherein said HDPE contains an ethylene homopolymer having a weight average molecular weight of 20000 to 40000 D.

3. A process as claimed in either of claims 1 and 2 wherein said HDPE has a density of 945 to 975 kg/m^3.

4. A process as claimed in any one of claims 1 to 3 wherein said HDPE has a density of 945 to 960 kg/m^3, a weight average molecular weight of 250000 to 350000 D, a number average molecular weight of 7000 to 18000 D, a molecular weight distribution of 18 to 50, an MFR_{21} of 3 to 8 g/10 min, a tensile modulus of at least 900 mPa, and a comonomer content of 1 to 2 wt. %.

5. A blow-moulded bimodal HDPE container having a volume of at least 2L, and an ESCR F_{50} of at least 500 hours.

6. A container as claimed in claim 5 having a volume of at least 8L.

7. A bimodal HDPE having the following characteristics:
a density of 940 to 970 kg/m^3;
a weight average molecular weight of 200000 to 450000 D;
a number average molecular weight of 6000 to 20000 D;
a molecular weight distribution of 15 to 55;
MFR_{21} of 2 to 12 g/10 min;
tensile modulus at least 900 mPa; and
a comonomer content of 0.5 to 10 wt%.